A STUDY ON EFFECT OF INDIAN HERBAL PREPARATIONS ON DNA DAMAGE AND ANTIOXIDANT PROFILE IN SEMINAL PLASMA OF INFERTILE MEN

ABSTRACT

OF THE

THESIS

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BY

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Male factor infertility remains a significant problem, contributing to ~ 50 % of the cases attending infertility clinics. Infertility is defined as failure of conception after at least 12 months of unprotected intercourse. In recent years, many studies have indicated a worldwide decreasing trend of male fertility in terms of average sperm counts and semen quality. A number of etiologies have been identified as potential causes of male infertility, which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction.

In recent years, concern has been expressed about the generation of reactive oxygen species (ROS) in the male reproductive tract. ROS are highly reactive molecules and they can oxidize lipids, amino acids and carbohydrates as well as may cause DNA mutations. Controlled production of free radical in body play an important positive role in the process of fertilization, redox signalling, cellular differentiation and organization, as also in programmed cell death (or apoptosis). Human spermatozoa have been demonstrated to have the capacity to produce ROS such as superoxide anion(O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). The generation of ROS has been shown to correlate with the fertilizing potential of human spermatozoa. ROS play dual role in spermatozoa. First, they are involved in their kinetic function which enables them to migrate through the female genital tract across the cumulus oophorus cells. Secondly, they are involved in their fusigenic function, which enables them to bind to zona pellucida and then fuse with the oocyte membrane. Normally, there is a fine balance between the amounts of ROS produced and scavenged by a cell. However, an excess of ROS have been implicated as an etiological factor of a very wide range of diseases.

Mammalian sperm cells contain highly specific lipids composition, high content of polyunsaturated fatty acids, plasmalogenes and sphingomyelins. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high level of phospholipids, sterols, saturated and polyunsaturated fatty acids, therefore, sperm cells are particularly susceptible to the damage induced by excessive ROS release. Peroxidation of polyunsaturated fatty acid has been implicated in a wide variety of pathological conditions including, cardiac and cerebral ischemic-reperfusion injury, and inflammatory joint diseases amongst others. The main product of lipid peroxidation is melondialdehyde (MDA). There are a lot of other products of lipid peroxidation such as: conjugated dienes, and secondary peroxidation products which include saturated and unsaturated hydrocarbons (e.g. ethane, petane and peroxides). Therefore, lipid peroxidation causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increase non-specific permeability of ions.

One area that has been studied intensely during the past decade as a cause for male infertility is the integrity of DNA in the nucleus of mature ejaculated spermatozoa. Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. Sperm DNA integrity is essential for accurate transmission of the genetic material to the offspring. Various studies have suggested that disturbances in the organization of the genomic material in sperm nuclei are negatively correlated with the fertility potential of spermatozoa, either in *vivo* or *in vitro.* Irregular strand breaks in sperm DNA and increased sensitivity of DNA to denaturation may be due to spermatogenic cells having undergone cell death by an apoptosis- like process to differing degrees during spermatogenesis.

Fragmented DNA, indicative of apoptosis, increase in ejaculated spermatozoa from infertile men and is one of the initial hallmarks of apoptosis, the most common form of eukaryotic cell death.

DNA fragmentation could be due to flaws in endogenous endonuclease activity resulting in DNA nicks or apoptosis that is mediated through Fas, a cell surface protein. Some reports have indicated that when >30% of sperm DNA is damaged, natural pregnancy is not possible. In addition, it has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis. Interest in the genomic integrity of the male gamete has intensified the growing concern about transmission of genetic diseases through intracytoplasmic sperm injection (ICSI). Recently, it has been suggested that, to assist in the risk assessment of ICSI, it would be appropriate to develop methods to measure DNA damage in the sperm and to correlate this with biological outcomes. This was attributed to the fact that conventional semen analysis does not adequately represent the diverse array of biological properties that the spermatozoon, as a highly specialized cell, expresses. This analysis may describe some aspects of the function of the testes and sperm, but do not explain the integrity of the male genome contained in the head of the sperm. In addition, the results of semen analysis can be very subjective and prone to both intra- and interobservervariability.

Specific and directed treatment for male infertility is not available due to unexplained and heterogeneous nature of the disorders. Under such circumstances, only assisted reproductive techniques are of some help. However, these treatments are expensive and inaccessible to all. The lack of available specific therapies for men with infertility demands the exploration of alternative therapies. Given the lack of

knowledge about etiological factors, a non-directed but general therapy may yield good results in a subcategory of patients. The rationale for the use of these therapies is based on the speculation that some forms of male infertility are caused by oxidative insult and hormonal imbalance and the use of alternative therapies may improve male fertility potential and semen quality. The later is also supported by our studies (Ahmad *et al.,* 2008, 2009; Shukla *et al.,* 2007, 2009: Mahdi *et al.,* 2009). In the Ayurveda and Unani systems of medicine practiced in India, several plants and plant products have been documented to fight against stress, impotence, infertility, and the aging process. Most of these plants are reported to be rich sources of anti-oxidants also. Given a significant role of oxidative stress in male infertility, these herbs bear tremendous potential in treating male infertility.

In view of above considerations, the present study was designed to investigate the semen, lipid and hormonal profile, oxidative biomarkers and DNA damage of infertile men. Moreover, the protective effects of some Indian medicines on aforementioned parameters were evaluated.

A) Evaluated following parameters:

1) To measure the Sperm count, motility, morphology etc.

2) To asses the levels of lipid profiles i.e. total lipids, cholesterol, triglycerides and phospholipids in seminal plasma of infertile men.

3) To estimate the levels of oxidative biomarkers i.e. lipid peroxides, protein carbonyl groups, total antioxidant capacity in seminal plasma and ROS in spermatozoa of infertile men.

4) To evaluate the activity of seminal antioxidant enzymes namely; superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase.

5) To asses the antioxidant vitamin A, E and C.

6) To estimate the levels of reproductive hormonl viz LH, FSH, Testosterone and Prolactin in serum of infertile men.

7) To asses the DNA damage in spermatozoa of infertile men.

B) To assess the protection afforded, if any, on the aforementioned parameters by the following Indian medicinal plants:

a) Mucuna pruriens

b) Withania somnifera

The Institutional Review Board and Ethics Committee of Chhatrapati Shahuji Maharaj (CSM) Medical University (Formerly- King George's Medical University), Lucknow, approved this study. The study population included control group (n = 100) and infertile patients (n = 100), aged 20-40 years, recruited from the Outpatient Department of Urology, CSM Medical University, Lucknow and infertile male partners of the couples attending the infertility clinic at the Department of Obstetric and Gynaecology, CSM. Medical University, Lucknow. The patients were further categorized in four groups according to semen parameters; normozoospermic infertile men (n = 25), had normal semen profile (defined below as in the control group) and infertility of unknown etiology, oligozoospermic infertile men (n = 25) had a sperm count <20 x 10^6/mL, motility >40%, and >40% normal morphology; and asthenozoospermic infertile men (n = 25) had a sperm count >20 x 10^6/mL, motility <40%, and >40% normal morphology and azoospermic (n = 25) had no sperm in ejaculated specimen or centrifuged pellets of specimens. The control group comprised of age-matched healthy men who had previously initiated at least one pregnancy and exhibited normal semen profile (sperm count >20 x 10^6/mL, motility >40%, and >40% normal morphology). Before enrolment in the study each subject's

informed written consent was obtained in response to a fully written and verbal explanation of the nature of study. The potential participants, each with infertility persisting longer than 1 year, were carefully examined with respect to physical, biochemical and semen parameters. As an exclusion criterion, the subjects having infection of accessory glands, diabetes, hypertension, arthritis, tuberculosis, or human immunodeficiency virus, those on drugs, or having other conditions known to influence oxidative stress and/or fertility were excluded. Additionally, medical histories of patients and their female partners were recorded. Infertile cases where a problem could be diagnosed in female partners were also excluded from the study. All subjects were instructed to continue normal diet without switching to dietary supplements during the course of treatment. The subjects were prescribed *M. pruriens* seed powder (5 g/day) and *W. somnifera* root powder (5 g/day) orally for 3 months with milk. This dosing schedule was as reported earlier by Singh (1974).

The results of our study demonstrated that male infertility is associated with abnormal semen profile i.e. low sperm count, motility and semen volume along with disturbance in the levels of seminal lipid profiles. We also observed that spermatozoa of infertile men produce significantly high levels of ROS and show more DNA damage as compared with fertile men. The same is reflected by elevated levels of seminal lipid peroxides, protein carbonyl groups and decreased seminal total antioxidant capacity (TAC). The condition was further complexed by reduced activity of antioxidant enzymes namely SOD, catalase, glutathione reductase and glutathione peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we also observed hormonal imbalance i.e., low level of testosterone and LH, and elevated levels of FSH and PRL, in all groups of infertile men.

We observed that treatment with *M. pruriens* and *W. somnifera* significantly improved sperm concentration and motility. Both the medicines improved the activity of antioxidant enzymes and recovered the levels of TAC, eventually reducing the levels of seminal lipid peroxides and protein carbonyl groups along with decreased the levels of ROS in spermatozoa in infertile men. Complementing our findings, earlier studies have reported that *M. pruriens* and *W. somnifera* inhibits lipid peroxidation in stress induced animals.

The detrimental effects of reactive oxygen species (ROS) on spermatozoa were suggested more than 65 years ago with the demonstration that exposure of sperm to oxygen results in sperm toxicity. It has been reported that semen of infertile males contains significantly high levels of ROS, as has been observed by us also, whereas fertile men do not have detectable levels of semen ROS. There are several reports that the ROS produced by leukocytes and/or by spermatozoa have deleterious effects on sperm function. The half-life of ROS is very short and it is difficult to detect ROS in semen directly. However, malondialdehyde (MDA), one of the lipid peroxidative end-products produced by ROS when it attacks sperm membrane, can indirectly reflect the damage of sperm. Therefore, the determination of MDA concentration in seminal plasma may be taken as one of important markers for the diagnosis and treatment of male infertility induced by excessive lipid peroxidation. We observed elevated ROS and lipid peroxide levels in all infertile men and also found that both the herbs used in the study (*M. pruriens* and *W. somnifera)* significantly decreased the lipid peroxides and MDA levels in infertile males. Along with the lipid peroxides and ROS, several studies have demonstrated that infertile men are more likely than fertile ones to have depressed TAC and lower levels of individual antioxidants. Moreover, our results showed that treatment with *M. pruriens* and *W. somnifera* significantly

decreased the levels of ROS in spermatozoa and seminal protein carbonyl groups along with increasing the levels of seminal TAC.

Our results showed a significant reduction in total lipid levels in all infertile groups as compared with controls. These results are similar to those reported earlier in chronic alcoholic infertile men, patients with chronic infections and infertile males. It may be suggested that an excess of ROS in infertile patients caused the oxidative degradation of seminal plasma lipids resulting in decreased level of lipids in these patients. It is well known that lipids in seminal plasma, apart from serving as energy source for spermatozoa during capacitation and fertilization process, also determine their structural integrity. There have been reports that cholesterol: phospholipids ratio influence the structural integrity and fluidity of membranes and increase in this ratio is known to be associated with decrease in fertility. A decrease in the phospholipid concentration with more or less unaltered cholesterol in seminal plasma, as reported here, may increase the cholesterol: phospholipid ratio in spermatozoa, which may also affect fertility.

Treatment with *M. pruriens* and *W. somnifera* significantly increased the levels of seminal plasma lipid profiles as compared to pre treatment groups. Both drugs possess potent antioxidant activity and we observed that treatment with these natural products in infertile patients inhibited the formation of lipid peroxides; this may have saved the seminal lipids, including triglycerides and phospholipids from breakdown.

In order to counteract the toxic effects of ROS, seminal plasma and spermatozoa are well endowed with an array of antioxidant mechanisms. The antioxidant enzymes; catalase, superoxide dismutase (SOD), glutathione peroxidase

and glutathione reductase have all been detected in seminal plasma and the activities of these antioxidant enzymes were found reduced in all groups of infertile men, and this may be mainly due to elevated levels of oxidative insult. However, treatment with *M. pruriens and W. somnifera* significantly improved the activity of all antioxidant enzymes. which may have eventually contributed in reducing the levels of lipid peroxides, spermatozoa ROS and protein carbonyl groups along with increase in the levels of TAC in infertile men. Interestingly, we also observed that after treatment with *M. pruriens* and *W. somnifera* the seminal plasma levels of vitamins A, C, and E were significantly increased in infertile men. This might have also contributed to the improvement in sperm concentration and motility. Vitamins A, C, and E are biological antioxidants that function as detoxifying agents, immunopotentiators, and immunoactivators. Earlier it has been reported that *M. pruriens and W. somnifera* both have strong antioxidant properties and this might be the reason, in the recovery of antioxidants levels in infertile men.

In the present study we observed that spermatozoa of infertile men not only produce significantly high levels of ROS, but they also showed more DNA damage as compared with fertile men. Moreover, our results also demonstrated that *M. pruriens* and *W. somnifera* were capable of reducing the ROS levels and seminal lipid peroxides, and this may have been the reason for the reduction in the extent of DNA damage in the spermatozoa of post-treated men. Earlier it has been reported that elevated levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men. Recently, a significant positive correlation between ROS and DNA fragmentation has been reported. Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of

modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements.

We observed decreased levels of LH and T and increased levels of FSH and PRL in men with poor semen quality. Moreover, we also found that treatment with *M. pruriens* significantly recovered the levels of testosterone and LH and suppressed FSH and PRL. Furthermore, it was seen that normozoospermic infertile men presented with less perturbed hormone levels and less DNA damage, despite having high ROS content. The reduced DNA damage may also probably be due to less hormonal imbalance seen in these men. However, oligozoopermic and asthenozoospermic infertile men exhibited more hormonal disbalance and high DNA damage, inspite of oxidative stress levels being similar to that of normozoospermic infertile men. This may indicate the important contribution of sex hormones in protecting against oxidative stress induced DNA damage. Our findings of correction in DNA damage of infertile men by *M. pruriens* is supported by a very recent study by Suresh *et al.,* (2009). They also reported that aged rats exhibited hypo-spermatogenesis, high DNA damage in sperms and had high overall oxidative stress. While treatment with *M. pruriens* lead to the improvement in spermatogenesis, reduction in ROS, spermatozoon DNA damage and overall oxidative stress.

On the basis of available literature and our observation we tried to figure out the possible mechanism of action for both the herbs. *M. pruriens* seeds are rich source of L-DOPA and its metabolites, which include epinephrine and norepinephrine. Though the mode of action of DOPA and catecholamine on human fertility is not yet established, it may be proposed that as *M. pruriens* contains high levels of L-DOPA, its metabolite, dopamine, may stimulate the hypothalamus and forebrain to secrete

Gonadotropin-releasing hormone (GnRH) which may further stimulate the anterior pituitary gland to secrete follicule stimulating hormone (FSH) and leutinizing hormone (LH) causing increased synthesis of testosterone by Leydig cells of the testis and this is elaborated in figure I (Ahmad *et al.*, 2008).

Apart of L-DOPA, *M. pruriens* contains many bioactive constituents, including alkaloids, coumarins, flavonoids, and alkylamines, which may play an important role in increasing the antioxidant capacity. There are also reports that the methanol extract of *M. pruriens* seeds has strong antioxidant activity, because it inhibits 1,1 diphenyl-2-picryl-hydrazyl and hydroxyl radical, scavenges nitric oxide and superoxide anion and reduces hydrogen peroxide. The presence of these compounds may be the reason for protective effects of *M. pruriens* against oxidative stress and DNA damage. According to our results, it may be safely concluded that treatment with *M. pruriens* exerted a potent restorative and invigorative effect in all groups of infertile males. Moreover, we also observed that infertile subjects (mainly oligozoospermic and normozoospermic, roughly 30%) were able to achieve pregnancy following treatment with *M. pruriens* (Mahdi, unpublished data).

The biological basis and exact mechanism of action of *W. somnifera* on infertility is not well known but previous experimental studies showed that treatment with aqueous extracts of *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats. It is reported that *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous

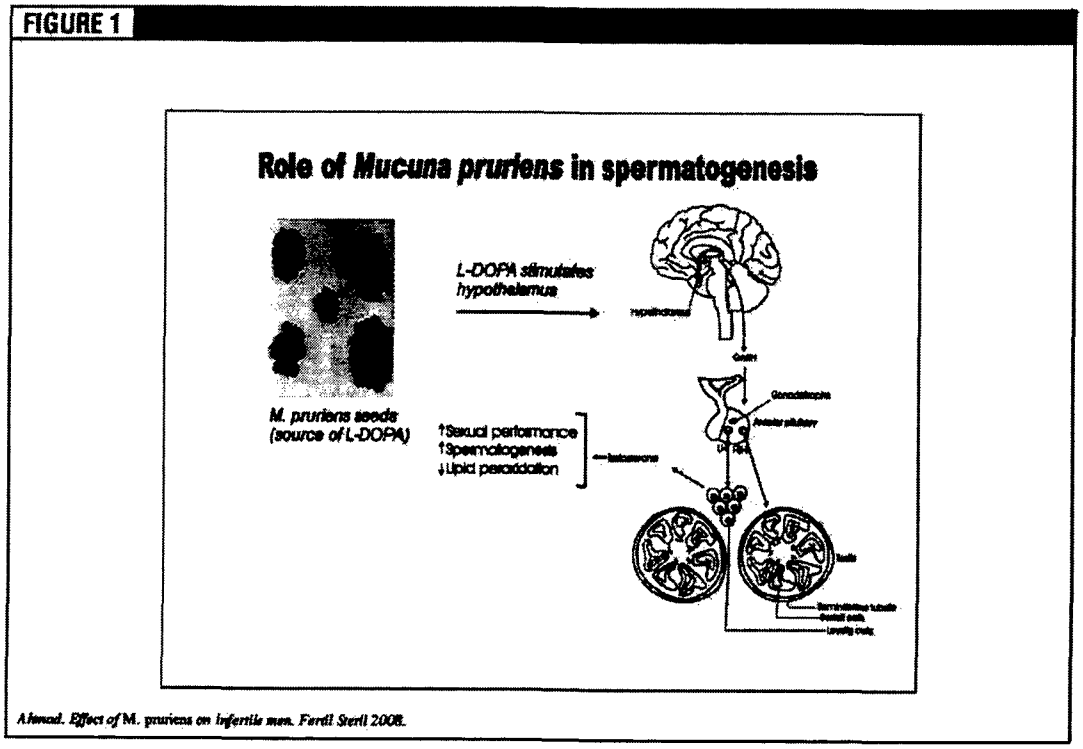

Figure I: Role of *M. pruriens* in spermatogenesis

tubules. Moreover, *W. somnifera* has also been reported to have several pharmacological effects e.g. antistressor; adaptogenic and cardioprotective properties etc. The roots of *W. somnifera* contain several alkaloids, withanolides, a few flavanoids and reducing sugars. More than twenty active constituents have been reported in roots of *W. somnifera* till date including withaferin A, sitoindosides VII–X, withanoside I–VII, choline, beta-sitosterol etc. The presence of these compounds may be the reason for diverse effects of *W. somnifera* on semen properties. However, the spermogenic and /or steroidogenic activity of any of these compounds, if any, has not been explored.

Concluding it may be stated that in the present study we observed significantly high levels of ROS in spermatozoa of infertile men alongwith elevated levels of lipid peroxides, protein carbonyl groups, decreased total antioxidant capacity and perturbed seminal lipid profiles. We also found suppressed activity of antioxidant enzymes

namely SOD, catalase, glutathione reductase and peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we observed hormonal imbalance and elevated spermatozoa DNA damage in all groups of infertile men, and this may be the reason for decrease in sperm concentration and motility. Our results showed that *M. pruriens* and *W. somnifera* were able to restore sex hormone levels, reduce oxidative stress and the extent of DNA damage along with the recovery of lipid profiles (Fig. II). Improved spermatogenesis and fertility as a result of *M. pruriens* administration could be attributed to the presence L-DOPA along with a number of other bioactive substances. Similarly, the positive effect of the roots of *W. somnifera* may be due to presence of several alkaloids, Withanolides and flavanoids in it.

Ours is the first report considering oxidative stress, DNA damage and hormonal imbalance along with the protective role of *M. pruriens* and *W. somnifera* in infertile human subjects, and the correlation in between. Although the efficacy of *M. pruriens* in improving male factor fertility was found more in comparison to *W. somnifera,* however, a mixture of the two herbs may be evaluated for better outcome. Further research on these herbs could focus on fractioning of the herbs and identification of the active constituents. This would not only help in understanding the basis of the treatment offered by these herbs but may also help in understanding the possible causes and novel pathways involved in fertility maintenance in humans. Alhough the desired effect of the herbs may not be achieved through purified components but it is still worth giving it a try.

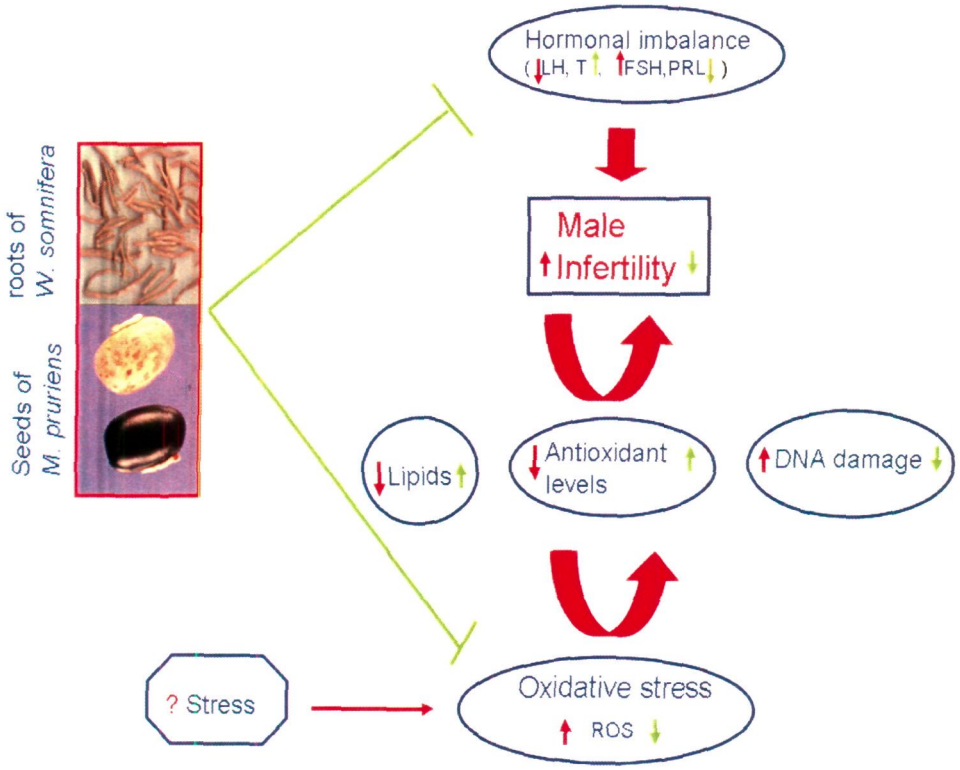

Figure 38: Proposed mechanism of action of *M. pruriens* and *W. somnifera* in preventing DNA damage and infertility. *M. pruriens* and *W. somnifera* restrains hormonal imbalance and oxidative stress to prevent infertility.

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Dated : .....

Approved : .....

.....

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CERTIFICATE

This is to certify that **Mr. Mohd Kaleem Ahmad** has carried out this work for Ph.D. Thesis on the topic entitled: "A Study on Effect of Indian Herbal Preparations on DNA Damage and Antioxidant Profile in Seminal Plasma of Infertile Men" under my supervision. To the best of my knowledge, this is Mr. Kaleem's original work which is suitable for the award of **Ph. D.** degree in **Biochemistry** of the Aligarh Muslim University, Aligarh, India.

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Date .....

CERTIFICATE

This is to certify that the research work incorporated in the thesis entitled: "A Study on Effect of Indian Herbal Preparations on DNA damage and Antioxidant Profile in Seminal Plasma of Infertile Men" has been carried out by **Mr. Mohd Kaleem Ahmad** in the Department of Biochemistry, *C. S. M. Medical University* (Formerly-King George's Medical University), Lucknow. In my belief this work is suitable for the award of the degree of **Doctor of Philosophy** by the Faculty of Medicine, J.N. Medical College, Aligarh Muslim University, Aligarh.

This is to further certify that the work embodied in this thesis is an original record of research work conducted by **Mr. Ahmad** in our department. The contents of this work have not yet been submitted in any form elsewhere.

Prof. A.A. Mahdi
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Dedicated to my dear parents as a token of love & appreciation

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(Mohd. Kaleem Ahmad)

CONTENTS

Abstract

Male factor infertility remains a significant problem, contributing to ~ 50 % of the cases attending infertility clinics. Infertility is defined as failure of conception after at least 12 months of unprotected intercourse. In recent years, many studies have indicated a worldwide decreasing trend of male fertility in terms of average sperm counts and semen quality. A number of etiologies have been identified as potential causes of male infertility, which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction.

In recent years, concern has been expressed about the generation of reactive oxygen species (ROS) in the male reproductive tract. ROS are highly reactive molecules and they can oxidize lipids, amino acids and carbohydrates as well as may cause DNA mutations. Controlled production of free radical in body play an important positive role in the process of fertilization, redox signalling, cellular differentiation and organization, as also in programmed cell death (or apoptosis). Human spermatozoa have been demonstrated to have the capacity to produce ROS such as superoxide anion(O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). The generation of ROS has been shown to correlate with the fertilizing potential of human spermatozoa. ROS play dual role in spermatozoa. First, they are involved in their kinetic function which enables them to migrate through the female genital tract across the cumulus oophorus cells. Secondly, they are involved in their fusigenic function, which enables them to bind to zona pellucida and then fuse with the oocyte membrane. Normally, there is a fine balance between the amounts of ROS produced and scavenged by a cell. However, an excess of ROS have been implicated as an etiological factor of a very wide range of diseases.

Mammalian sperm cells contain highly specific lipids composition, high content of polyunsaturated fatty acids, plasmalogenes and sphingomyelins. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high level of phospholipids, sterols, saturated and polyunsaturated fatty acids, therefore, sperm cells are particularly susceptible to the damage induced by excessive ROS release. Peroxidation of polyunsaturated fatty acid has been implicated in a wide variety of pathological conditions including, cardiac and cerebral ischemic-reperfusion injury, and inflammatory joint diseases amongst others. The main product of lipid peroxidation is melondialdehyde (MDA). There are a lot of other products of lipid peroxidation such as: conjugated dienes, and secondary peroxidation products which include saturated and unsaturated hydrocarbons (e.g. ethane, petane and peroxides). Therefore, lipid peroxidation causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increase non-specific permeability of ions.

One area that has been studied intensely during the past decade as a cause for male infertility is the integrity of DNA in the nucleus of mature ejaculated spermatozoa. Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. Sperm DNA integrity is essential for accurate transmission of the genetic material to the offspring. Various studies have suggested that disturbances in the organization of the genomic material in sperm nuclei are negatively correlated with the fertility potential of spermatozoa, either in *vivo* or *in vitro*. Irregular strand breaks in sperm DNA and increased sensitivity of DNA to denaturation may be due to spermatogenic cells having undergone cell death by an apoptosis- like process to differing degrees during spermatogenesis.

Fragmented DNA, indicative of apoptosis, increase in ejaculated spermatozoa from infertile men and is one of the initial hallmarks of apoptosis, the most common form of eukaryotic cell death.

DNA fragmentation could be due to flaws in endogenous endonuclease activity resulting in DNA nicks or apoptosis that is mediated through Fas, a cell surface protein. Some reports have indicated that when >30% of sperm DNA is damaged, natural pregnancy is not possible. In addition, it has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis. Interest in the genomic integrity of the male gamete has intensified the growing concern about transmission of genetic diseases through intracytoplasmic sperm injection (ICSI). Recently, it has been suggested that, to assist in the risk assessment of ICSI, it would be appropriate to develop methods to measure DNA damage in the sperm and to correlate this with biological outcomes. This was attributed to the fact that conventional semen analysis does not adequately represent the diverse array of biological properties that the spermatozoon, as a highly specialized cell, expresses. This analysis may describe some aspects of the function of the testes and sperm, but do not explain the integrity of the male genome contained in the head of the sperm. In addition, the results of semen analysis can be very subjective and prone to both intra- and interobservervariability.

Specific and directed treatment for male infertility is not available due to unexplained and heterogeneous nature of the disorders. Under such circumstances, only assisted reproductive techniques are of some help. However, these treatments are expensive and inaccessible to all. The lack of available specific therapies for men with infertility demands the exploration of alternative therapies. Given the lack of

knowledge about etiological factors, a non-directed but general therapy may yield good results in a subcategory of patients. The rationale for the use of these therapies is based on the speculation that some forms of male infertility are caused by oxidative insult and hormonal imbalance and the use of alternative therapies may improve male fertility potential and semen quality. The later is also supported by our studies (Ahmad *et al.,* 2008, 2009; Shukla *et al.,* 2007, 2009: Mahdi *et al.,* 2009). In the Ayurveda and Unani systems of medicine practiced in India, several plants and plant products have been documented to fight against stress, impotence, infertility, and the aging process. Most of these plants are reported to be rich sources of anti-oxidants also. Given a significant role of oxidative stress in male infertility, these herbs bear tremendous potential in treating male infertility.

In view of above considerations, the present study was designed to investigate the semen, lipid and hormonal profile, oxidative biomarkers and DNA damage of infertile men. Moreover, the protective effects of some Indian medicines on aforementioned parameters were evaluated.

A) Evaluated following parameters:

1) To measure the Sperm count, motility, morphology etc.

2) To asses the levels of lipid profiles i.e. total lipids, cholesterol, triglycerides and phospholipids in seminal plasma of infertile men.

3) To estimate the levels of oxidative biomarkers i.e. lipid peroxides, protein carbonyl groups, total antioxidant capacity in seminal plasma and ROS in spermatozoa of infertile men.

4) To evaluate the activity of seminal antioxidant enzymes namely; superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase.

5) To asses the antioxidant vitamin A, E and C.

6) To estimate the levels of reproductive hormonl viz LH, FSH, Testosterone and Prolactin in serum of infertile men.

7) To asses the DNA damage in spermatozoa of infertile men.

B) To assess the protection afforded, if any, on the aforementioned parameters by the following Indian medicinal plants:

a) Mucuna pruriens

b) Withania somnifera

The Institutional Review Board and Ethics Committee of Chhatrapati Shahuji Maharaj (CSM) Medical University (Formerly- King George's Medical University), Lucknow, approved this study. The study population included control group (n = 100) and infertile patients (n = 100), aged 20-40 years, recruited from the Outpatient Department of Urology, CSM Medical University, Lucknow and infertile male partners of the couples attending the infertility clinic at the Department of Obstetric and Gynaecology, CSM. Medical University, Lucknow. The patients were further categorized in four groups according to semen parameters; normozoospermic infertile men (n = 25), had normal semen profile (defined below as in the control group) and infertility of unknown etiology, oligozoospermic infertile men (n = 25) had a sperm count <20 x 10^6/mL, motility >40%, and >40% normal morphology; and asthenozoospermic infertile men (n = 25) had a sperm count >20 x 10^6/mL, motility <40%, and >40% normal morphology and azoospermic (n = 25) had no sperm in ejaculated specimen or centrifuged pellets of specimens. The control group comprised of age-matched healthy men who had previously initiated at least one pregnancy and exhibited normal semen profile (sperm count >20 x 10^6/mL, motility >40%, and >40% normal morphology). Before enrolment in the study each subject's

informed written consent was obtained in response to a fully written and verbal explanation of the nature of study. The potential participants, each with infertility persisting longer than 1 year, were carefully examined with respect to physical, biochemical and semen parameters. As an exclusion criterion, the subjects having infection of accessory glands, diabetes, hypertension, arthritis, tuberculosis, or human immunodeficiency virus, those on drugs, or having other conditions known to influence oxidative stress and/or fertility were excluded. Additionally, medical histories of patients and their female partners were recorded. Infertile cases where a problem could be diagnosed in female partners were also excluded from the study. All subjects were instructed to continue normal diet without switching to dietary supplements during the course of treatment. The subjects were prescribed *M. pruriens* seed powder (5 g/day) and *W. somnifera* root powder (5 g/day) orally for 3 months with milk. This dosing schedule was as reported earlier by Singh (1974).

The results of our study demonstrated that male infertility is associated with abnormal semen profile i.e. low sperm count, motility and semen volume along with disturbance in the levels of seminal lipid profiles. We also observed that spermatozoa of infertile men produce significantly high levels of ROS and show more DNA damage as compared with fertile men. The same is reflected by elevated levels of seminal lipid peroxides, protein carbonyl groups and decreased seminal total antioxidant capacity (TAC). The condition was further complexed by reduced activity of antioxidant enzymes namely SOD, catalase, glutathione reductase and glutathione peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we also observed hormonal imbalance i.e., low level of testosterone and LH, and elevated levels of FSH and PRL, in all groups of infertile men.

We observed that treatment with *M. pruriens* and *W. somnifera* significantly improved sperm concentration and motility. Both the medicines improved the activity of antioxidant enzymes and recovered the levels of TAC, eventually reducing the levels of seminal lipid peroxides and protein carbonyl groups along with decreased the levels of ROS in spermatozoa in infertile men. Complementing our findings, earlier studies have reported that *M. pruriens* and *W. somnifera* inhibits lipid peroxidation in stress induced animals.

The detrimental effects of reactive oxygen species (ROS) on spermatozoa were suggested more than 65 years ago with the demonstration that exposure of sperm to oxygen results in sperm toxicity. It has been reported that semen of infertile males contains significantly high levels of ROS, as has been observed by us also, whereas fertile men do not have detectable levels of semen ROS. There are several reports that the ROS produced by leukocytes and/or by spermatozoa have deleterious effects on sperm function. The half-life of ROS is very short and it is difficult to detect ROS in semen directly. However, malondialdehyde (MDA), one of the lipid peroxidative end-products produced by ROS when it attacks sperm membrane, can indirectly reflect the damage of sperm. Therefore, the determination of MDA concentration in seminal plasma may be taken as one of important markers for the diagnosis and treatment of male infertility induced by excessive lipid peroxidation. We observed elevated ROS and lipid peroxide levels in all infertile men and also found that both the herbs used in the study (*M. pruriens* and *W. somnifera)* significantly decreased the lipid peroxides and MDA levels in infertile males. Along with the lipid peroxides and ROS, several studies have demonstrated that infertile men are more likely than fertile ones to have depressed TAC and lower levels of individual antioxidants. Moreover, our results showed that treatment with *M. pruriens* and *W. somnifera* significantly

decreased the levels of ROS in spermatozoa and seminal protein carbonyl groups along with increasing the levels of seminal TAC.

Our results showed a significant reduction in total lipid levels in all infertile groups as compared with controls. These results are similar to those reported earlier in chronic alcoholic infertile men, patients with chronic infections and infertile males. It may be suggested that an excess of ROS in infertile patients caused the oxidative degradation of seminal plasma lipids resulting in decreased level of lipids in these patients. It is well known that lipids in seminal plasma, apart from serving as energy source for spermatozoa during capacitation and fertilization process, also determine their structural integrity. There have been reports that cholesterol: phospholipids ratio influence the structural integrity and fluidity of membranes and increase in this ratio is known to be associated with decrease in fertility. A decrease in the phospholipid concentration with more or less unaltered cholesterol in seminal plasma, as reported here, may increase the cholesterol: phospholipid ratio in spermatozoa, which may also affect fertility.

Treatment with *M. pruriens* and *W. somnifera* significantly increased the levels of seminal plasma lipid profiles as compared to pre treatment groups. Both drugs possess potent antioxidant activity and we observed that treatment with these natural products in infertile patients inhibited the formation of lipid peroxides; this may have saved the seminal lipids, including triglycerides and phospholipids from breakdown.

In order to counteract the toxic effects of ROS, seminal plasma and spermatozoa are well endowed with an array of antioxidant mechanisms. The antioxidant enzymes; catalase, superoxide dismutase (SOD), glutathione peroxidase

and glutathione reductase have all been detected in seminal plasma and the activities of these antioxidant enzymes were found reduced in all groups of infertile men, and this may be mainly due to elevated levels of oxidative insult. However, treatment with *M. pruriens and W. somnifera* significantly improved the activity of all antioxidant enzymes. which may have eventually contributed in reducing the levels of lipid peroxides, spermatozoa ROS and protein carbonyl groups along with increase in the levels of TAC in infertile men. Interestingly, we also observed that after treatment with *M. pruriens* and *W. somnifera* the seminal plasma levels of vitamins A, C, and E were significantly increased in infertile men. This might have also contributed to the improvement in sperm concentration and motility. Vitamins A, C, and E are biological antioxidants that function as detoxifying agents, immunopotentiators, and immunoactivators. Earlier it has been reported that *M. pruriens and W. somnifera* both have strong antioxidant properties and this might be the reason, in the recovery of antioxidants levels in infertile men.

In the present study we observed that spermatozoa of infertile men not only produce significantly high levels of ROS, but they also showed more DNA damage as compared with fertile men. Moreover, our results also demonstrated that *M. pruriens* and *W. somnifera* were capable of reducing the ROS levels and seminal lipid peroxides, and this may have been the reason for the reduction in the extent of DNA damage in the spermatozoa of post-treated men. Earlier it has been reported that elevated levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men. Recently, a significant positive correlation between ROS and DNA fragmentation has been reported. Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of

modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements.

We observed decreased levels of LH and T and increased levels of FSH and PRL in men with poor semen quality. Moreover, we also found that treatment with *M. pruriens* significantly recovered the levels of testosterone and LH and suppressed FSH and PRL. Furthermore, it was seen that normozoospermic infertile men presented with less perturbed hormone levels and less DNA damage, despite having high ROS content. The reduced DNA damage may also probably be due to less hormonal imbalance seen in these men. However, oligozoopermic and asthenozoospermic infertile men exhibited more hormonal disbalance and high DNA damage, inspite of oxidative stress levels being similar to that of normozoospermic infertile men. This may indicate the important contribution of sex hormones in protecting against oxidative stress induced DNA damage. Our findings of correction in DNA damage of infertile men by *M. pruriens* is supported by a very recent study by Suresh *et al.,* (2009). They also reported that aged rats exhibited hypo-spermatogenesis, high DNA damage in sperms and had high overall oxidative stress. While treatment with *M. pruriens* lead to the improvement in spermatogenesis, reduction in ROS, spermatozoon DNA damage and overall oxidative stress.

On the basis of available literature and our observation we tried to figure out the possible mechanism of action for both the herbs. *M. pruriens* seeds are rich source of L-DOPA and its metabolites, which include epinephrine and norepinephrine. Though the mode of action of DOPA and catecholamine on human fertility is not yet established, it may be proposed that as *M. pruriens* contains high levels of L-DOPA, its metabolite, dopamine, may stimulate the hypothalamus and forebrain to secrete

Gonadotropin-releasing hormone (GnRH) which may further stimulate the anterior pituitary gland to secrete follicule stimulating hormone (FSH) and leutinizing hormone (LH) causing increased synthesis of testosterone by Leydig cells of the testis and this is elaborated in figure I (Ahmad *et al.*, 2008).

Apart of L-DOPA, *M. pruriens* contains many bioactive constituents, including alkaloids, coumarins, flavonoids, and alkylamines, which may play an important role in increasing the antioxidant capacity. There are also reports that the methanol extract of *M. pruriens* seeds has strong antioxidant activity, because it inhibits 1,1 diphenyl-2-picryl-hydrazyl and hydroxyl radical, scavenges nitric oxide and superoxide anion and reduces hydrogen peroxide. The presence of these compounds may be the reason for protective effects of *M. pruriens* against oxidative stress and DNA damage. According to our results, it may be safely concluded that treatment with *M. pruriens* exerted a potent restorative and invigorative effect in all groups of infertile males. Moreover, we also observed that infertile subjects (mainly oligozoospermic and normozoospermic, roughly 30%) were able to achieve pregnancy following treatment with *M. pruriens* (Mahdi, unpublished data).

The biological basis and exact mechanism of action of *W. somnifera* on infertility is not well known but previous experimental studies showed that treatment with aqueous extracts of *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats. It is reported that *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous

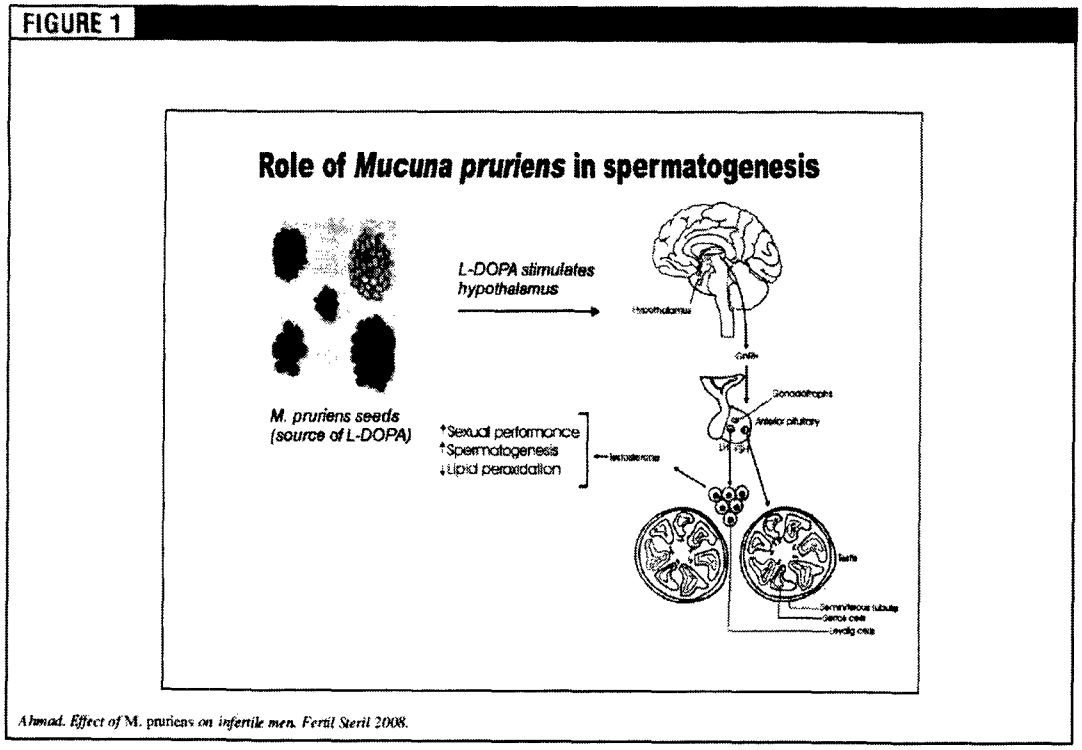

Figure I: Role of *M. pruriens* in spermatogenesis

tubules. Moreover, *W. somnifera* has also been reported to have several pharmacological effects e.g. antistressor; adaptogenic and cardioprotective properties etc. The roots of *W. somnifera* contain several alkaloids, withanolides, a few flavanoids and reducing sugars. More than twenty active constituents have been reported in roots of *W. somnifera* till date including withaferin A, sitoindosides VII–X, withanoside I–VII, choline, beta-sitosterol etc. The presence of these compounds may be the reason for diverse effects of *W. somnifera* on semen properties. However, the spermogenic and /or steroidogenic activity of any of these compounds, if any, has not been explored.

Concluding it may be stated that in the present study we observed significantly high levels of ROS in spermatozoa of infertile men alongwith elevated levels of lipid peroxides, protein carbonyl groups, decreased total antioxidant capacity and perturbed seminal lipid profiles. We also found suppressed activity of antioxidant enzymes

namely SOD, catalase, glutathione reductase and peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we observed hormonal imbalance and elevated spermatozoa DNA damage in all groups of infertile men, and this may be the reason for decrease in sperm concentration and motility. Our results showed that *M. pruriens* and *W. somnifera* were able to restore sex hormone levels, reduce oxidative stress and the extent of DNA damage along with the recovery of lipid profiles (Fig. II). Improved spermatogenesis and fertility as a result of *M. pruriens* administration could be attributed to the presence L-DOPA along with a number of other bioactive substances. Similarly, the positive effect of the roots of *W. somnifera* may be due to presence of several alkaloids, Withanolides and flavanoids in it.

Ours is the first report considering oxidative stress, DNA damage and hormonal imbalance along with the protective role of *M. pruriens* and *W. somnifera* in infertile human subjects, and the correlation in between. Although the efficacy of *M. pruriens* in improving male factor fertility was found more in comparison to *W. somnifera,* however, a mixture of the two herbs may be evaluated for better outcome. Further research on these herbs could focus on fractioning of the herbs and identification of the active constituents. This would not only help in understanding the basis of the treatment offered by these herbs but may also help in understanding the possible causes and novel pathways involved in fertility maintenance in humans. Alhough the desired effect of the herbs may not be achieved through purified components but it is still worth giving it a try.

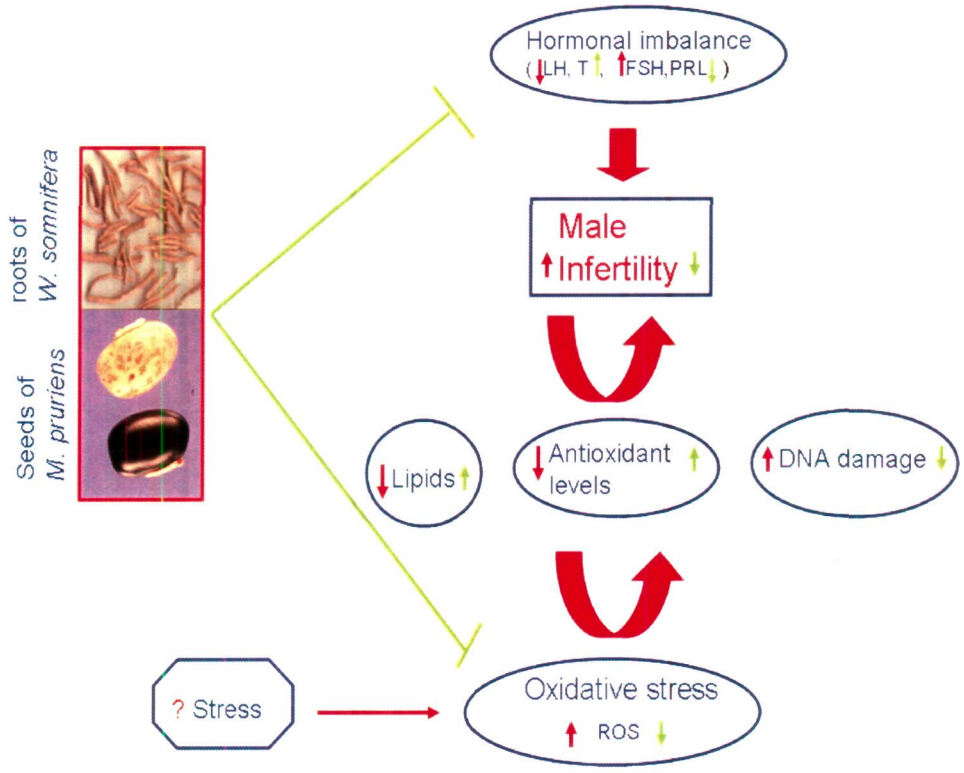

Figure 38: Proposed mechanism of action of *M. pruriens* and *W. somnifera* in preventing DNA damage and infertility. *M. pruriens* and *W. somnifera* restrains hormonal imbalance and oxidative stress to prevent infertility.

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Abbreviations

ABTS	: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA	: Analysis Of Variance
ATP	: Adinosine triphosphate
BSA	: Bovine serum albumin
°C	: Degree Celsius
cAMP	: Cyclic Adensine
CAT	: Catalase
cGMP	: Cyclic guanosine monophosphate
cm	: Centimeter
DCFDA	: Dichlorofluorescein
DNPH	: Dinitrophenyl hydrazine
DTNB	: 5, 5'-Dithio-bisl-2-nitorbenzoic acid
EDTA	: Ethylene diamine tetra acetic acid
ELISA	: Enzyme-linked immunosorbent assay
$Fecl_3$	: Ferric chloride
FSH	: Follicle stimulating hormone
GPx	: Gluthathione peroxidase
GR	: Glutathione reductase
GSH	: Glutathione
GSSG	: Oxidized Glutathione
H^+	: Hydrogen ion
H_2O_2	: Hydrogen peroxide
HCl	: Hydrogen chloride

HPLC	: High pressure liquid chromatography
K	: Potassium chloride
K^+	: Potassium ion
L	: Litre
LH	: Luteinizing hormone
LPO	: Lipid peroxidation
M	: Molar
mA	: milli Amphere
MDA	: Malondialdehyde
mg	: milligram
MgCl	: Magnesium chloride
min	: minute
ml	: milliliter
mm	: millimetre
mM	: millimolar
MW	: molecular weight
Na	: Sodium ion
NaCl	: Sodium Chloride
NADH	: Nicotinamide adenine clinucleotide
NBT	: Nitroblue Tetrazolium
ng	: nano gram
nM	: Nanomolar
NO	: Nitrate
NO^-	: Nitrite ion
O^{2-}	: Superoxide radical

OH^-	: Hydroxyl radical
OTM	: Olive Tail Movement
PBS	: Phosphate buffered saline
PMS	: Phenozine mehtosulpahte
PRL	: Prolactin
ROS	: Reactive oxygen species
RT	: Room temperature
S.E.M.	: Standard Error of the Mean
SDS	: Sodium dodecyl sulphate
SOD	: Superoxide dismutase
T	: Testosterone
TAC	: Total antioxidat capacity
TBA	: Thioberbituric acid
U	: Units
µg	: Microgram
µl	: micro litre
V	: Volt

Introduction

1. MALE INFERTILITY

Male infertility is a multifactorial disease process with a number of potential contributing causes. Male factors contribute to almost 50% of cases of infertility; in the remainder, it may be due to either a female factor or a combination of male and female factors (Gopalkrisnan *et al.,* 1996). The World Health Organization (WHO) defines infertility as the inability of a couple to conceive after one year of regular unprotected intercourse (WHO, 1995). A substantial number of couples seek fertility treatment because of poor semen quality, and there is evidence in the literature that male reproductive function seems to have deteriorated considerably in the past 4 to 5 decades. Carlsen *et al.,* (1992) observed a significant decline in mean sperm concentration from $113x10^6$ /ml in 1940 to $66x10^6$/ml in 1990, or 0.94 x 10^6/ml/year. A number of etiologies have been identified as potential causes of male infertility (Fig. 1), which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction (Ollero *et al.,* 2001).

Important issues related to the evaluation of the male factor include the most appropriate time for the male evaluation, the most efficient format for a comprehensive male examination, and definition of rationale and effective medical and surgical regimens in the treatment of these disorders. It is extremely important in the evaluation of infertility to consider the couple as a unit in evaluation and treatment and to proceed in a parallel investigative manner until a problem is uncovered. It has been shown that the longer a couple remains subfertile, the worse their chance for an effective cure. Many couples experience significant apprehension and anxiety after only a few months of failure to conceive. Unduly prolonged unprotected intercourse should not be advocated before a workup of the man is instituted. Initial screening of

the man should be considered whenever the patient presents with the chief complaint of infertility. This initial evaluation should be rapid, non-invasive and cost effective. Of interest is the fact that pregnancy rates of up to 50% have been reported when only the woman has been investigated and treated even when the man was found to have moderately severe abnormalities of semen quality.

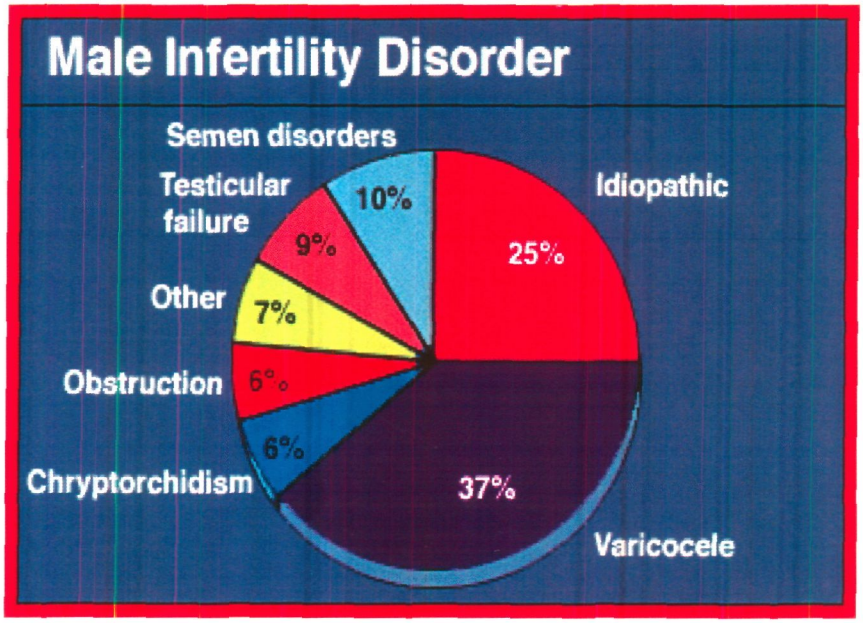


Figure 1. Etiology of male infertility

1.1 Spermatozoa

The mature sperm cell (spermatozoa) (Fig. 2) is 0.05 millilitres long. It consists of a head, body and tail. The head is covered by the acrosome cap and contains a nucleus of dense genetic material from the 23 chromosomes. It is attached from the neck to the body containing mitochondria that supply energy for the sperm's activity. The tail is made of protein fibers that contract on alternative sides, giving a characteristic wave like movement that drives the sperm through the seminal fluid, this also supplies additional energy.

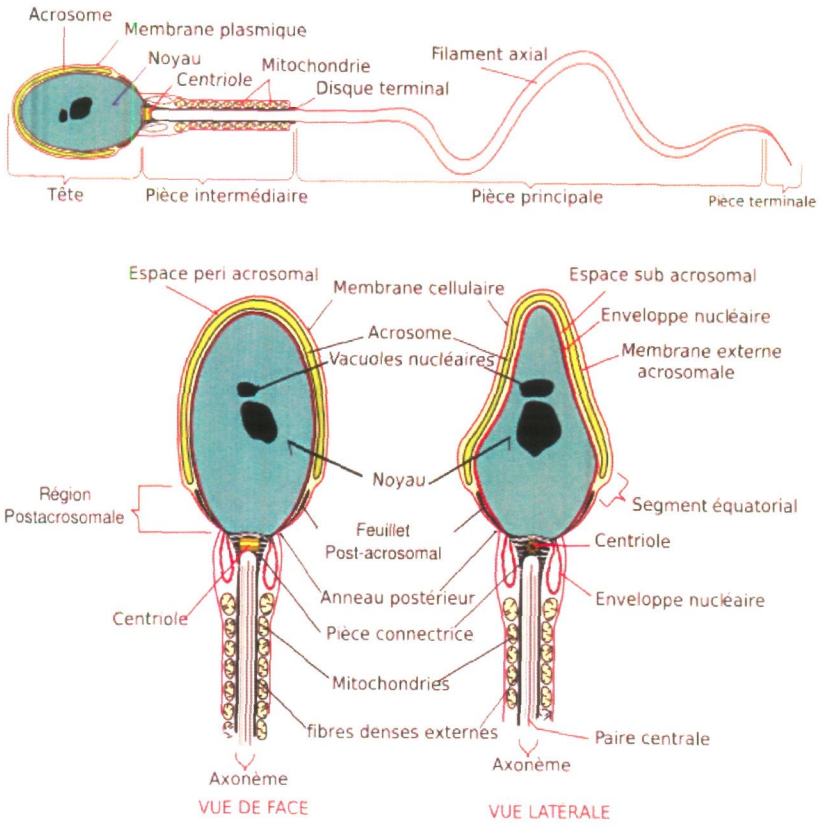


Figure 2. Structure of mature spermatozoa

Some sperm have two heads or two tails and if the testes are too warm they may die or spermatogenesis may not occur. Sperm swim at a rate of about 1 to 4 mm (0.12 inches) per minute. That is an average and it is different for every man.

Sperm cells are made in the testes where it takes about 72 days for one sperm to grow. Sperm production requires a temperature which is three to five degrees below body temperature. The scrotum has a built-in thermostat, which keeps the sperm at the correct temperature while they are being stored. Sperm can develop at the temperature they need i.e. 95° - 97° F or 35° to 36° C.

1.2. Causes of Male Infertility

More than 90% of male infertility cases are due to low sperm counts, poor sperm quality, or both. The remaining cases of male infertility can be caused by a number of factors including anatomical problems, hormonal imbalances, and genetic defects.

1.2.1. *Sperm Abnormalities*

Sperm abnormalities can be caused by a range of factors, including congenital birth defects, diseases, chemical exposure, and lifestyle habits. In many cases, the causes of sperm abnormalities are unknown. Sperm abnormalities are categorized by whether they affect sperm count, sperm movement, or sperm shape. They include:

1.2.1.1. *Low Sperm Count (Oligospermia).* Low sperm count is one of the main causes of male infertility. It is considered that a man has low sperm count when he has less than 20 million spermatozoa per ml of ejaculate. Several medical conditions as well as many biologic and environmental factors may cause low sperm count, temporarily or permanently. Unfortunately, not many proper treatments for increasing sperm count are available. Some commonly used medications and supplements for increasing sperm count are testosterone, certain vitamins, high-protein diets, anti-oxidants, and herbal semen enhancement pills.

1.2.1.2. No Sperm (Azoospermia). It refers to the complete absence of sperm cells in the ejaculate, and accounts for 10 - 15% of cases of male infertility. Partial obstruction anywhere in the long passages through which sperm pass can reduce sperm counts. Sperm count varies widely over time, and temporary low counts are common. Therefore, a single test that reports a low count may not be a representative result.

1.2.1.3. *Poor Sperm Motility (Asthenospermia).* Sperm motility is the sperm's ability to move. If movement is slow, not in a straight line, or both, the sperm have difficulty invading the cervical mucous or penetrating the hard outer shell of the egg. If 60% or more of sperm have normal motility, the sperm is at least average in quality. If less than 40% of sperm are able to move in a straight line, the condition is considered abnormal. Sperm that move sluggishly may have genetic or other defects that render them incapable of fertilizing the egg. Poor sperm motility may be associated with DNA fragmentation and may increase the risk for passing on genetic diseases.

1.2.1.4. *Abnormal Sperm Morphology (Teratospermia).* Morphology refers to shape and structure. Abnormally shaped sperm cannot fertilize an egg. About 60% of the sperm should be normal in size and shape for adequate fertility. The perfect sperm structure is an oval head and long tail.

1.2.2. *Retrograde Ejaculation*

Retrograde ejaculation occurs when the muscles of the bladder wall do not function properly during orgasm and sperm are forced backward into the bladder instead of moving forward out of the urethra. Sperm quality is often impaired. Retrograde ejaculation can be the consequence of several conditions:

Surgery to the lower part of the bladder or prostate (the most common cause of retrograde ejaculation)

Diseases such as diabetes and multiple sclerosis

Spinal cord injury or surgery

Medications such as tranquilizers, certain antipsychotics, or blood pressure medications also may cause temporary retrograde ejaculation.

Aging

1.2.3. *Structural Abnormalities*

Any structural abnormalities that damage or block the testes, tubes, or other reproductive structures can have a profound effect on fertility.

1.2.3.1. *Cryptorchidism.* Cryptorchidism is a condition usually seen in newborn infants in which the testicles fail to descend from the abdomen into the scrotum. Cryptorchidism is associated with mild to severe impairment of sperm production.

1.2.3.2. *Hypospadias.* This is a birth defect in which the urinary opening is on the underside of the penis, can prevent sperm from reaching the cervix if not surgically corrected.

1.2.3.3. *Blockage in the Tubes that Transport Sperm.* Some men are born with a blockage in the epididymis or ejaculatory ducts or other problems that later affect fertility. Some men lack the vas deferens, the tube that carries sperm from the testicles out through the penis. Low semen levels in ejaculate may be associated with structural abnormalities in the tubes transporting the sperm.

1.2.4 *Hormonal Factors*

1.2.4.1. *Hypothalamic Disease*

Kallmann's syndrome which is an isolated gonadotropin (LH and FSH) deficiency occurs in both a sporadic and familial form and although uncommon i.e. 1 in 10,000 men, it is second to Klinefelter's syndrome as a cause of hypogonadism. The syndrome is often associated with anosmia, congenital deafness, hair lip, cleft palate, craniofacial asymmetry, renal abnormalities, color blindness. The hypothalamic hormone gonadotrophin releasing hormone (GnRH) appears to be absent. If exogenous GnRH is administered, both LH and FSH are released from the pituitary. Except for the gonadotropin deficiency, anterior pituitary function is intact. The syndrome appears to be inherited either as an autosomal recessive trait or an

autosomal dominant trait with incomplete penetrance. The differential diagnosis should include delayed puberty. Kallmann's syndrome main distinguishing features though are testes less than 2 cm in diameter and positive family history with the presence of anosmia. "Fertile eunuch" are individuals with isolated LH deficiency. They have eunuchoid proportions with variable degrees of virilization and gynecomastia. They characteristically have large testes and semen containing a few sperm. Plasma FSH levels are normal but both the serum LH and testosterone concentrations are low to normal. The cause appears to be a partial gonadotropin deficiency in which there is adequate LH to stimulate testosterone production with resultant spermatogenesis but insufficient testosterone to promote virilization. In isolated FSH deficiency which is rare, patient's are normally virilized and have normal testicular size and baseline levels of LH and testosterone. Sperm counts range from zero to a few sperm. Serum FSH levels are low and do not respond to GnRH stimulation. Congenital hypogonadotropic syndromes are associated with secondary hypogonadism and a multitude of other somatic findings. Prader-Willi syndrome is characterized by hypogonadism, hypomentia, hypotonia at birth and obesity. Laurence-Moon-Bardet-Biedel syndrome is an autosomal recessive trait characterized by mental retardation, retinitis pigmentosa, polydactyly and hypogonadism. All these syndromes are reported to be due to a defect in hypothalamic deficiency of GnRH (Kottler *et al.,* 2004).

1.2.4.2. *Pituitary Disease*

Pituitary insufficiency may result from tumors, infarctions, iatrogenic causes like surgery and radiation or one of several infiltrative processes. If pituitary insufficiency occurs prior to puberty, growth retardation associated with adrenal and thyroid deficiency is the major clinical presentation. Hypogonadism that occurs in a sexually

mature male usually has its origin in a pituitary tumor. Decreasing libido, impotence and infertility may occur years before symptoms of an expanding tumor i.e. such as headaches, visual abnormalities, or thyroid/adrenal hormone deficiency. Once an individual has passed through normal puberty, it takes a long time for secondary sexual characteristics to disappear unless adrenal insufficiency is present. The testes will eventually become small and soft. The diagnosis is made by low serum testosterone levels with low or low normal plasma gonadotropins concentrations. Depending on the degree of panhypopituitarism, plasma corticosteroids will be reduced along with plasma TSH and growth hormone levels.

1.2.4.3. *Hyperprolactinemia*

Hyperprolactinemia can cause both reproductive and sexual dysfunction. Prolactin-secreting tumors of the pituitary gland whether from a microadenoma (less than 10 mm) or a macroadenoma, can result in loss of libido, impotence, galactorrhea, gynecomastia and altered spermatogenesis. Patients with a macroadenoma usually present with visual field abnormalities and headaches. These patients are reported have low serum testosterone levels but basal serum levels of LH and FSH are either low or low normal and reflect an inadequate pituitary response to depressed testosterone.

Approximately 80% of men with hemochromatosis have testicular dysfunction. Their hypogonadism may be secondary to iron deposition in the liver or may be primarily testicular as a result of iron deposition in the testes. Iron deposits have also been found in the pituitary, implicating this gland as the major site of abnormality

1.2.5. *Genetic Factors*

There is increasing evidence that genetics play a key role in the development of male infertility, in particular, spermatogenic disorders and spermatogenic failure. It is estimated that in men presenting with male infertility, around 30% may be related to azoospermia or oligoasthenoteratoazoospermia (Kupker *et al.,* 1999). Of these, 48-50% may be of a nonobstruction form. As many as 13-20% of men with azoospermia /oligospermia present with microdeletion of the distal end of the Y chromosome, the so–called AZF region (azoospermia factor). This has been subdivided into three main overlapping regions; *AZFa, AZFb* and *AZFc.* Of these the most common microdeletions involve the *AZFc* region which has copies of the *DAZ* (deleted in azoospermia) gene. Abnormalities of the candidate genes for spermatogenesis in autosomal chromosomes such as the homologous gene to DAZ mapped from chromosome 3 in man, is the *DAZLI* (DAZ-like). Additional gene families have been implicated as having an absolute requirement for successful spermatogenesis, these include the RBM (RNA-binding protein) located within the AZF locus for (azoospermia factor), also *DBY* (dead box on the Y), *DFFRY* (Drosophila fatfacet-related Y) (Vogt *et al.,* 1998).

The development of azoospermia may also be related to structural chromosomal abnormalities such as translocations or rearrangement of chromosomes. In this case, sections of chromosomes are translocated to other sections, rendering arms of shorter length involving both the X and Y chromosomes. X-autosome and Y-chromosome translocations give rise to spermatogenic impairment. Additional genetic diseases may contribute to reported cases of azoospermia include genetic mutation of androgen receptor genes (Kupker *et al.,* 1999). For successful spermatogenesis, testicular descent and normal male sexual differentiation, androgen stimulation via a

functional androgen receptor is necessary. Androgen receptor mutations have been reported as a potential source of interference for continual receptor function. Dowsing *et al.,* (1999) reviewed the genetic complement of the receptor, findings indicated that an inherited defect may be a potential source of infertility in men.

1.2.6. *Occupational Factors*

Occupational exposure to heavy metals; cadmium, lead, zinc and arsenic have been reported to impair spermatogenesis (Coste *et al.,* 1991). In addition, the effects of specific herbicides and pesticides have also been reported to be toxic to spermatogenesis (Eaton *et al.,* 1986). Smoking has also been linked with lower sperm concentrations; however, correlations are difficult to develop as supporting evidence is sketchy (Joffe & Li, 1994).

1.2.7. *Environmental Causes*

Heat exposure has been reported to have adverse consequences for spermatogenesis mainly reducing semen quality, increasing the incidence of congenital malformations and increasing the incidence of testicular cancer (Mieusset and Bujan, 1995).

1.2.8. *Systemic and Iatrogenic Causes*

Medical disorders associated with male infertility directly include; chromosomal disorders, testicular maldescent, Kartagner's syndrome, Cystic fibrosis, androgen receptor deficiency and coeliac disease. Acquired disorders can also have an effect on male fertility which include the following:

Endocrine Diseases – thyrotoxicosis, diabetes, hepatic failure, renal failure, pituitary failure.

Respiratory diseases – bronchiectasis, sinusitis and bronchitis.

Neurological diseases- paraplegia, myotonic dystrophy.

1.2.9. *Infections -associated with male infertility*

Microbial infections have been associated with male infertility for many years. Gomez *et al.,* (1979) reported colonisation to human sperm by *Neisseria gonorrhoea. Chlamydia trachomatis* is known *to* cause urethritis and epididymitis in men. Moss *et al.,* (1986) discovered IgM antibodies of the D-K range to Chlamydia in serum from sperm donors. Dieterle *et al.,* (1995) also detected *C. trachomatis* by semen analysis and by serum IgG and IgA analysis.

Infertility in males has also been associated with male accessory gland infections due to mumps, tuberculosis and syphilis. Leukocytospermia (raised WBC > 1 x106 ml in semen) has been associated with infections of the epididymis and accessory sexual glands by *C. trachomatis* and *Ureaplasma urealytium.*

Examination of these accessory sexual glands is difficult especially as extensive pathological changes in the sex glands may often be present without clear symptoms. Assessment of WBC-specific reactive oxygen species aids diagnosis of leukocytospermia (Vicari, 1999).

Immunological causes reflect the presence of anti-sperm antibodies (IgA and IgG) which have been associated with lower sperm motility. Although the relationship with infertile couples has been noted, antibodies to sperm surface antigens are also found in fertile control populations who may conceive, but at a lower rate (Busacca *et al.,* 1989).

2. OVERVIEW ON BIOCHEMISTRY OF OXIDATIVE STRESS IN SEMEN

ROS are products of normal cellular metabolism. Most of the body's energy is produced by the enzymatically controlled reaction of oxygen with hydrogen in

oxidative phosphorylation occurring within the mitochondria during oxidative metabolism. During this enzymatic reduction of oxygen to produce energy, free radicals are formed (Valko *et al.,* 2007). A free radical is defined as a molecule containing one or more unpaired electrons in atomic or molecular orbitals. The addition of one electron to dioxygen (O_2) forms the superoxide anion radical ($O_2^{\cdot-}$), the primary form of ROS. This superoxide anion can then be directly or indirectly (enzymatic, metal catalyzed) converted to secondary ROS such as the hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$) or hydrogen peroxide (H_2O_2). The terms free radical and ROS are commonly used in an interchangeable manner, despite the fact that not all ROS are free radicals (Cheeseman and Slater, 1993). For example, hydrogen peroxide (H_2O_2) is considered a ROS but it is not a free radical since it does not contain unpaired electrons. In addition, there is a sub-class of free radicals derived from nitrogen which includes nitrous oxide, peroxynitrite, nitroxyl anion and peroxynitrous acid. Free radicals seek to participate in chemical reactions that relieve them of their unpaired electron, resulting in the oxidation of lipids in membranes, amino acids in proteins and carbohydrates within nucleic acids (Fig.3) (Ochsendorf, 1999).

Within semen there are two principal sources of production of free radicals; leukocytes and the sperm itself. The vast majority of semen specimens contain leukocytes, with neutrophils being the predominant leukocyte type (Aitken *et al.,* 1994; Aitken *et al.,* 1995). As the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. However, a link between the

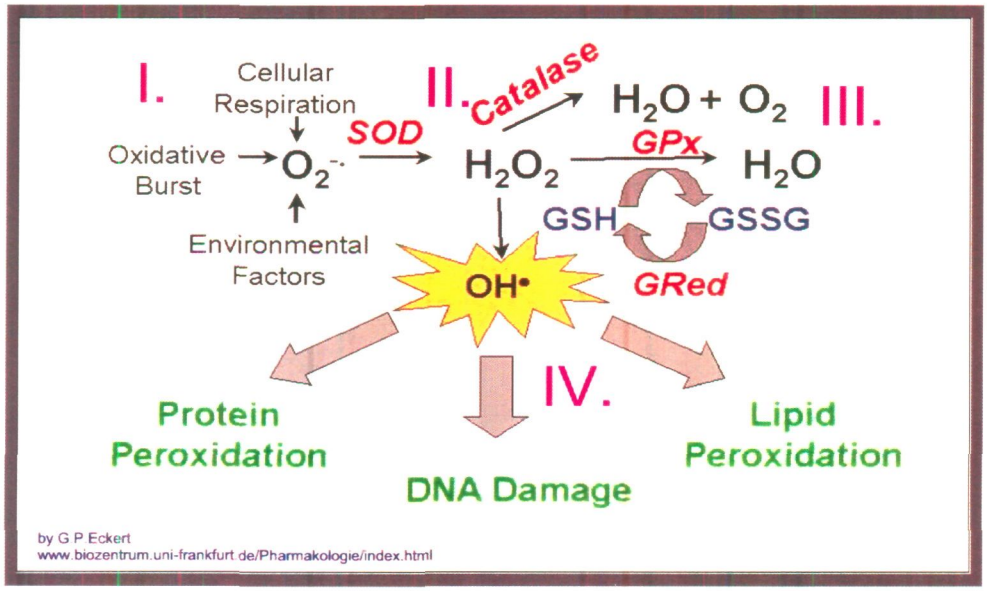


Figure 3. ROS mediated damage in spermatozoa

presence of leukocytes in semen and male oxidative infertility is still under debate (Wolff, 1995). Several researchers have reported a positive correlation between seminal leukocyte numbers and ROS production (Aitken *et al.*, 1994; Whittington *et al.*, 1999; Sharma *et al.*, 2001). However, other studies have failed to find a significant difference in seminal leukocyte concentration between fertile and infertile men (Christiansen *et al.*, 1991; Tomlinson *et al.*, 1993; Aitken *et al.*, 1995; Rodin *et al.*, 2003), and the activation state of leukocytes must also play an important role in determining final ROS output. This is supported by the observation of a positive correlation between seminal ROS production and pro-inflammatory seminal plasma cytokines such as interleukin IL-6 (Camejo *et al.*, 2001; Nandipati *et al.*, 2005), IL-8 (Rajasekaran *et al.*, 1995; Martinez *et al.*, 2007) and tumour necrosis factor TNFα (Sanocka *et al.*, 2003; Martinez *et al.*, 2007).

Every human ejaculate contains leukocytes which make the quantification of spermatozoa-specific ROS production more complex. However, sperm isolation techniques have been used to confirm that spermatozoa themselves are responsible for some ROS generation, not just contaminating seminal leukocytes (Baker *et al.*, 2003).

Separation of sperms from seminal leukocytes using density-gradient centrifugation has shown the 'sperm fraction' to produce significant ROS. As this fraction may still contain a very low number of leukocytes, experiments have been conducted where leukocytes are further depleted using magnetic beads coated with leukocyte-specific CD45 antibodies (Aitken *et al.,* 1996). After removing all detectable leukocyte contamination, ROS production can still be recorded, confirming the ability of sperm to generate ROS. The relative importance of sperm and leukocyte production of ROS varies between individuals but can be estimated using the leukocyte specific activator, N-formyl-methionine-leucine-phenylalanine (FMLP).

The ability of sperm to produce ROS inversely correlates with their maturational stage. During spermatogenesis there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form. Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece. These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of b-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt. NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane (Gomez *et al.,* 1996; Fisher and Aitken, 1997; Said *et al.,* 2005). As a result, teratozoospermic sperm produce increased amounts of ROS compared with morphologically normal sperm.

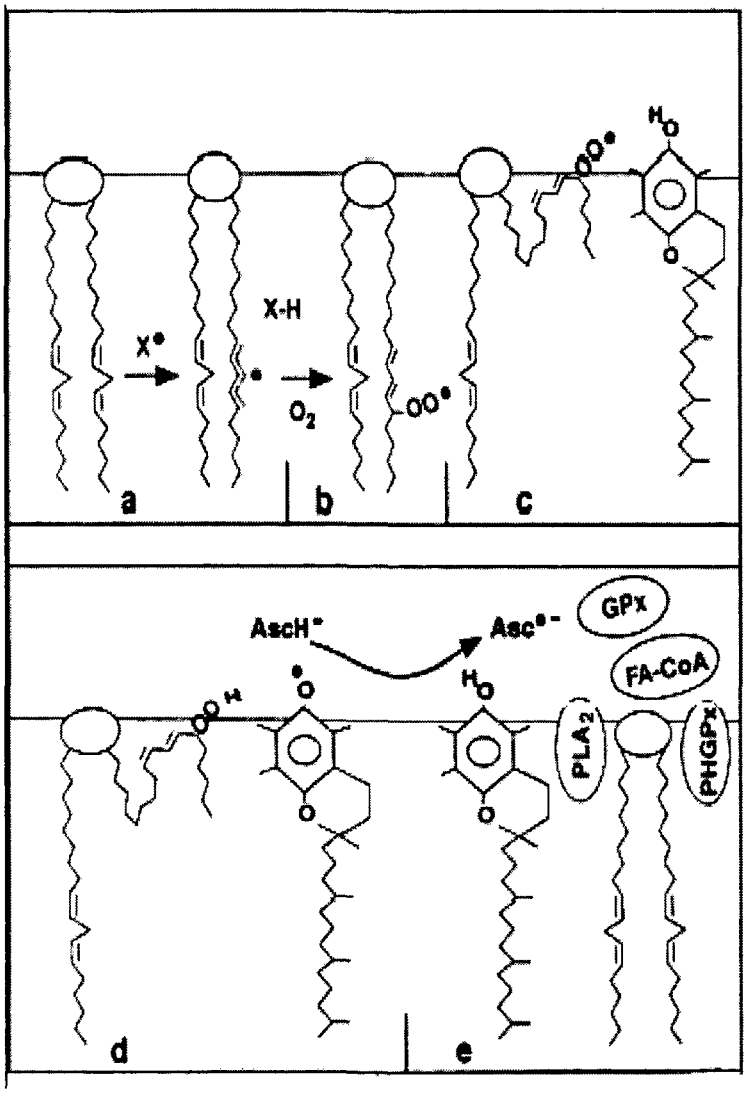
The relative importance of leukocytes and sperm in the aetiology of oxidative stress is currently under debate. The rate of production of ROS by leukocytes is reported to be 1000 times higher than that of spermatozoa at capacitation (Plante *et al.,* 1994), making leukocytes the likely dominant producer of seminal ROS. When seminal ROS production is divided into that produced by the sperm themselves

(intrinsic ROS) and that made by the leukocytes (extrinsic), an interesting observation is seen (Henkel *et al.,* 2005). While both intrinsic and extrinsic ROS production is negatively correlated with sperm DNA integrity, the relationship is significantly stronger for intrinsic ROS production. This suggests that while leukocytes produce more ROS than sperm on a per cell basis, the close proximity between intrinsic ROS production and sperm DNA makes intrinsic ROS production a more important variable in terms of fertility potential.

2.1. Lipid Peroxidation of Sperm Plasma Membrane

Lipid peroxidation (LPO) is broadly defined as ''oxidative deterioration of PUFA'' (ie, fatty acids that contain more than two carbon-carbon double bonds; Halliwell, 1984). The LPO cascade occurs in two fundamental stages: initiation and propagation. The hydroxyl radical (OHx) is a powerful initiator of LPO (Aitken and Fisher, 1994). Most membrane PUFAs have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene C-H bonds weaker and, therefore, hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the rearrangement of the double bonds, which forms a conjugated diene radical that can then be oxidized. This means that lipids, which contain many methylene-interrupted double bonds, are particularly susceptible to peroxidation (Blake *et al.*, 1987). Conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical (ROOx), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid hydroperoxides are stable under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxides to generate

alkoxyl and peroxyl radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell (Halliwell, 1984).



(a) Initiation of the peroxidation process by an oxidizing radical X ˙ , by abstraction of a hydrogen atom, thereby forming a pentadienyl radical. (b) Oxygenation to form a peroxyl radical and a conjugated diene. (c) Peroxyl radical moiety partitions to the water-membrane interface where it is poised for repair by tocopherol. (d) Peroxyl radical is converted to a lipid hydroperoxide, and the resulting tocopherol radical can be repaired by ascorbate. (e) Tocopherol has been recycled by ascorbate; the resulting ascorbate radical can be recycled by enzyme systems. The enzymes phospholipase A2 (PLA2), phospholipid hydroperoxide glutathione peroxidase (PH-GPx), glutathione peroxidase (GPx) and fatty acyl-coenzyme A (FA-CoA) cooperate to detoxify and repair the oxidized fatty acid chain of the phospholipid. (from Buettner 1993).

Figure 4. Mechanism of Lipid Peroxidation

Propagation of LPO depends on the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malondialdehyde (Fig.4). This byproduct has been used in biochemical assays to monitor the degree of peroxidative damage in spermatozoa (Aitken *et al.*, 1989; Aitken and Fisher, 1994). The results of such an assay exhibit an excellent correlation

with the degree to which sperm function is impaired in terms of motility and the capacity for sperm- oocyte fusion (Aitken *et al.*, 1993; Sidhu *et al.,* 1998).

2.2. Impairment of Sperm Motility

The increased formation of ROS has been correlated with a reduction of sperm motility (Aitken *et al.*, 1989; Iwasaki and Gagnon, 1992; Lenzi *et al.*, 1993; Agarwal *et al.*, 1994a; Armstrong *et al.,* 1999). The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (de Lamirande and Gagnon, 1995). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken *et al.*, 1997). Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione and low glutathione content. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids (Griveau *et al.,* 1995).

2.3. Effect of Sperm Morphology on ROS Production

Gomez *et al.,* (1998) have indicated that levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen. The link between poor semen quality and increased ROS generation lies in the

presence of excess residual cytoplasm (cytoplasmic droplet). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective (Huszar *et al.,* 1997). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme G6PD (Aitken, 1999). Recent studies by Ollero *et al.,* (2001) and Gil-Guzman *et al.,* (2001) have shown that levels of ROS production in semen were negatively correlated with the percentage of normal sperm forms as determined by World Health Organization (WHO, 1999) classification and by Kruger's strict criteria (Kruger *et al.,* 1987). The correlation of seminal ROS levels with morphologically abnormal sperm was also evident when morphology slides were scored using a sperm deformity index (SDI) (r 5 .31; P 5 .01;). The SDI was introduced by Aziz *et al.,* (1996) as a novel expression of sperm morphological abnormalities that were found to be highly correlated with fertilization in vitro. This new method for sperm morphology assessment uses a multiple entry scoring technique in which an abnormal sperm is classified more than once if more than one deformity is observed. SDI is calculated by dividing the total number of deformities observed by the number of sperm randomly selected and evaluated, irrespective of their morphological normality. SDI was significantly higher in a group of infertile men who had high levels of seminal ROS than in a group of infertile men with low ROS and in a group of fertile sperm donors (Mahfouz *et al.,* 2009). The link between seminal ROS and high SDI may be causal and related to the greater capacity of morphologically abnormal spermatozoa to produce ROS and this currently underway for further investigating. However, this new finding may have significant

implications for evaluating the fertility potential of sperms both in vivo and in vitro. Excessive production of ROS by sperms in patients with leukocytospermia implies that both the free-radical generating sperm themselves and any normal sperm in the immediate vicinity will be susceptible to oxidative damage. Furthermore, once the process of LPO is initiated, the self-propagating nature of this process ensures a progressive spread of the damage throughout the sperm population.

2.4. Mechanism of Antioxidant Protection in Semen

The human body has developed several antioxidant strategies to protect itself from ROS damage. This allows for normal oxidative metabolism to occur without damaging the cells, while still allowing for normal ROS-mediated cellular responses such as destruction of infectious pathogens and intracellular signalling (Valko *et al.*, 2007). Oxidative stress occurs when the production of ROS overwhelms the antioxidant defense mechanisms leading to cellular damage (Fig.5). Seminal plasma and sperm themselves are well endowed with an array of protective antioxidants (Fujii *et al.*, 2003; Garrido *et al.*, 2004a). Superoxide dismutase (SOD) and catalase are enzymatic antioxidants which inactivate the superoxide anion ($O_2^{\cdot -}$) and peroxide (H_2O_2) radicals by converting them into water and oxygen. SOD is present within both sperm and seminal plasma (Mennella and Jones, 1980; Zini *et al.*, 1993). The addition of SOD to sperm in culture has been confirmed to protect them from oxidative attack (Kobayashi *et al.*, 1991). While some investigators have reported minor reductions in seminal plasma SOD activity in infertile men (Alkan *et al.*, 1997; Sanocka *et al.*, 1997), many have not (Miesel *et al.*, 1997; Zini *et al.*, 2000; Hsieh *et al.*, 2002). However, the majority of evidence does support a link between deficient seminal catalase activity and male infertility (Jeulin *et al.*, 1989; Alkan *et al.*, 1997; Miesel *et al.*, 1997; Sanocka *et al.*, 1997; Zini *et al.*, 2000). Glutathione peroxidase

(GPx) is the final member of the seminal enzymatic antioxidant triad. GPx consists of a family of antioxidants (GPx1-5) that are involved in the reduction of hydroperoxides using glutathione as an electron donor. The GPxs are located within the testis, prostate, seminal vesicles, vas deferens, epididymis, seminal plasma and spermatozoa themselves (reviewed by Vernet *et al.*, 2004).

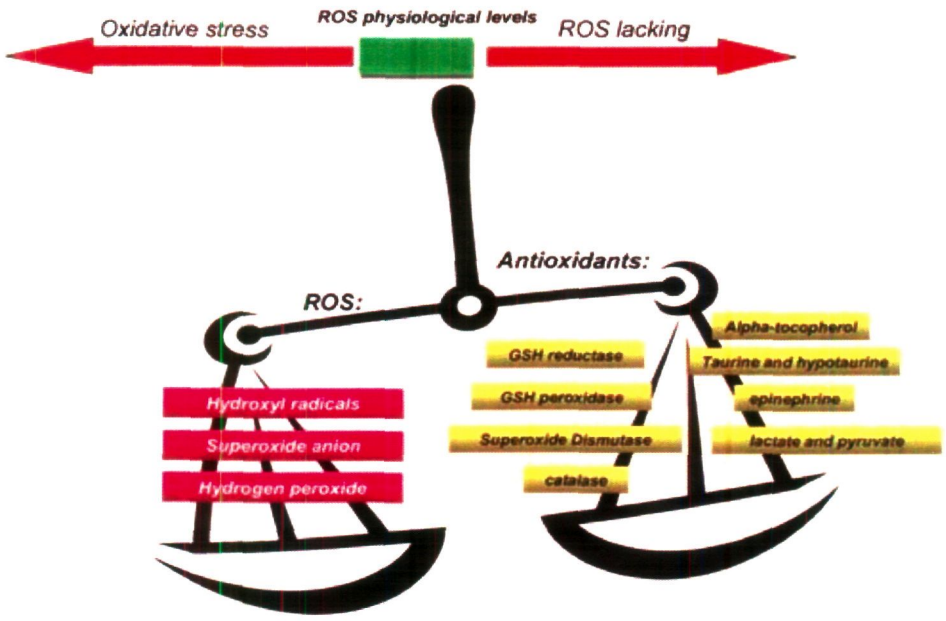


Figure 5. Imbalance between Oxidants and Antioxidants (Oxidative Stress)

GPx play an important protective role against oxidative attack since its specific inhibition in vitro using mercaptosuccinate leads to a large increase in sperm lipid peroxidation (Twigg *et al.*, 1998). Male factor infertility has been linked with a reduction in seminal plasma (Giannattasio *et al.*, 2002) and spermatozoa (Garrido *et al.*, 2004b) GPx activity, further supporting an important role for this enzyme in male fertility. In addition, men exhibiting leukospermia-associated oxidative stress have been reported to have significantly reduced GPx activity within their spermatozoa (Therond *et al.*, 1996). Finally, the continued activity of GPx depends on the regeneration of reduced glutathione by glutathione reductase (GR). Selective inhibition of GR reduces the availability of reduced glutathione for maintaining GPx

activity, thereby exposing sperms to oxidative stress (Williams and Ford, 2004). The coordinated activity of GPx, GR and glutathione clearly play a pivotal role in protecting sperm from oxidative attack.

The non-enzymatic antioxidants present within semen include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate and prostasomes. These agents principally act by directly neutralizing free radical activity chemically. However, they also provide protection against free radical attack by two other mechanisms. Albumin can intercept free radicals by becoming oxidized itself, thereby sparing sperm from attack (Twigg *et al.,* 1998). Alternatively, extracellular organelles (prostasomes) secreted by the prostate have been shown to fuse with leukocytes within semen and reduce their production of free radicals (Skibinski *et al.,* 1992; Saez *et al.,* 1998). A substantial number of researchers have reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile compared with fertile men (Fraga *et al.,* 1991; Fraga *et al.,* 1996; Smith *et al.,* 1996; Therond *et al.,* 1996; Lewis *et al.,* 1997; Gurbuz *et al.,* 2003; Koca *et al.,* 2003; Mostafa *et al.,* 2006; Song *et al.,* 2006).

Antioxidants contained within seminal plasma are obviously helpful for preventing sperm oxidative attack following ejaculation. However, during spermatogenesis and epididymal storage, the sperms are not in contact with seminal plasma antioxidants and must rely on epididymal/testicular antioxidants and their own intrinsic antioxidant capacity for protection. Sperms are therefore vulnerable to oxidative damage during epididymal transit, especially when there is epididymal inflammation such as male genital tract infection. In addition, testicular biopsies from men with varicocele-associated oxidative stress have shown an increase in oxidative

DNA damage within spermatogonia and spermatocytes (Ishikawa *et al.,* 2007). Therefore, while seminal plasma antioxidants may help minimize ejaculated sperm oxidative stress, they have no capacity to prevent oxidative damage initiated 'up stream' at the level of the testis and epididymis.

Ascorbic acid is a water-soluble ROS scavenger with high potency. In seminal plasma, ascorbic acid concentrations are 10-fold higher than in serum (Dawson *et al.,* 1987; Jacob *et al.,* 1992). In semen sample exhibiting ROS activity, ascorbate concentrations in the seminal plasma are significantly reduced (Lewis *et al.,* 1997). With a pharmacological supplementation of vitamin C (1g/day), a more than 2-fold increase in plasma ascorbic acid concentration can be achieved (Wen *et al.,* 1997). Moreover, ascorbic acid concentration in seminal plasma is also positively related to the percentage of morphologically normal spermatozoa. It has been suggested that ascorbic acid is a protected vitamin in the epididymis (Thiele *et al.,* 1995). Furthermore, it has been shown that ascorbic acid protects human spermatozoa against endogenous oxidative DNA damage (Fraga *et al.,* 1991). The amount of DNA damage is significantly greater in infertile male patients with low levels of ascorbic acid in their semen than in control patients (Kodama *et al.,* 1997).

There are a number of reports on supplementation studies with vitamin C in humans using biomarker of oxidative damage to DNA, lipids (lipid oxidation releases mutagenic aldehydes), and proteins. Though there are positive and negative studies, if the fact that the blood cell saturation occurs at about 100mg/day (Kallner *et al.,* 1979). is taken as consideration, then the evidence suggests that this level minimizes DNA damage (Duthie *et al.,* 1996).

Studies have shown that concentration of ascorbic acid in seminal plasma directly reflects dietary intake, and lower levels of vitamin C may lead to infertility and

increased damage to the sperm genetic material (Dabrowski and Cierezko, 1996). Fraga *et al.*, (1991) demonstrated this by reducing ascorbic acid intake in healthy men from 250 mg to 5 mg per day. Seminal plasma levels of vitamin C decreased by 50 percent, with a concomitant 91 percent increase in sperm DNA damage. Supplementing vitamin C improves the quality of sperm in smokers (Dawson *et al.*, 1992). When sperm stick together (a condition called agglutination), fertility is reduced. Vitamin C reduced sperm agglutination (Dawson *et al.*, 1983), and supplementation with 200-1000mg per day increased the fertility of men with this condition in a controlled study (Dawson *et al.*, 1990).

Vitamin E, the major fat-soluble antioxidant, is consumed primarily from dietary vegetable oils and nuts. α-tocopherol (vitamin E) is lipid soluble and acts mainly with in cell membranes (Ford and Whittington, 1998). Vitamin E is a well-documented antioxidant and has been shown to inhibit free radical induced damage to sensitive cell membranes (Aitken *et al.*, 1989). Vitamin E deficiency in animals has been known to cause infertility (Thiessen *et al.*, 1975). In a preliminary human trial 100-200 IU of vitamin E given daily to both partners of infertile couples led to a significant increase in fertility (Bayer R., 1960). Vitamin E supplementation may enhance fertility by decreasing free radical damage to sperm cells. In another preliminary study, men with low fertilization rates in previous attempts, at *in-vitro* fertilization, were given 200 IU of vitamin E per day for three months (Geva *et al.*, 1996). After one month of supplementation, fertilization rates increase significantly, and the amount of oxidative stress on sperm cell decreased. However, the evidence in favor of vitamin E remains preliminary. A review of research on vitamin E for male infertility concluded that there is no justification for its use in treating this condition

(Martin *et al.,* 1998). Controlled trails are needed to validate these promising preliminary findings.

In one study, lipid peroxidation in the seminal plasma and spermatozoa was estimated by malondialdehyde (MDA) concentrations. Oral supplementation with vitamin E significantly decreased MDA concentration and improved sperm motility, resulting in a 21 percent pregnancy during the study (Suleiman *et al.,* 1996). In one randomized, cross-over, controlled trial, 600 mg/day vitamin E improved sperm function in the zona binding assay, therefore enhancing the ability of the sperm to penetrate the egg in vitro (Kessopoulou *et al.,* 1995).

2.5. Seminal free radicals—friend or foe?

Sperm were the first type of cell reported to produce free radicals. In this pioneering studies, MacLeod (1943) noted that incubation of sperm under conditions of high oxygen tension lead to a rapid loss of their motility. The addition of the antioxidant catalase to the medium preserved sperm motility, prompting MacLeod to suggest that sperm must produce hydrogen peroxide during normal oxidative metabolism. Since this publication, it has evolved that three inter-related mechanisms account for oxidative stress-mediated male infertility—impaired motility, impaired fertilization and oxidative DNA damage.

The underlying pathology behind free radicals ability to reduce sperm motility was first reported by Jones *et al.,* (1979). They reported that ROS-induced peroxidation of the sperm membrane decreases its flexibility and therefore tail motion. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids. Direct ROS damage to mitochondria, decreasing energy availability, may also impede sperm motility (de Lamirande and Gagnon, 1992; de Lamirande *et al.,* 1997, 1998). By either mechanism, oxidative stress impairs

sperm motility and will result in less sperm reaching the oocyte for fertilization (Whittington *et al.*, 1999; Kao *et al.*, 2008).

Low level production of free radicals by sperm plays a positive role in preparation for fertilization (capacitation). Hydrogen peroxide stimulates the acrosome reaction and sperm hyperactivation (de Lamirande and Gagnon, 1993), thereby assisting the sperm's transit through the cumulus and zona pellucida. Low concentrations of hydrogen peroxide also cause tyrosine phosphorylation, which augments sperm membrane binding to the zona pellucida ZP-3 protein (Aitken *et al.*, 1995b), ultimately boosting sperm–oocyte fusion (Aitken *et al.*, 1998). However, high levels of ROS production lead to peroxidation of the sperm acrosomal membrane and diminished acrosin activity (Zalata *et al.*, 2004), and impaired sperm–oocyte fusion (Aitken *et al.*, 1989; Ichikawa *et al.*, 1999; Saleh *et al.*, 2003a, b; Zorn *et al.*, 2003a; Jedrzejczak *et al.*, 2005).

Free radicals have the ability to directly damage sperm DNA by attacking the purine and pyrimidine bases and the deoxyribose backbone. Normally, sperm DNA is tightly packaged by protamines protecting it from free radical attack. However, infertile men often exhibit deficient protamination, leaving the sperm DNA particularly vulnerable to ROS attack (Oliva, 2006). Alternatively, free radicals can initiate apoptosis within the sperm, leading to caspase-mediated enzymatic degradation of the DNA (Kemal Duru *et al.*, 2000; Wang *et al.*, 2003; Moustafa *et al.*, 2004; Villegas *et al.*, 2005). Several investigators (Kodama *et al.*, 1997; Aitken *et al.*, 1998; Saleh *et al.*, 2002b; Oger *et al.*, 2003; Wang *et al.*, 2003; Henkel *et al.*, 2005; Kao *et al.*, 2008) have now confirmed the link between oxidative stress and sperm DNA damage using various techniques such as terminal deoxynucleotidyl transferase-

mediated dUTP nick-end labeling (TUNEL), sperm chromatin structure assay (SCSA) and measurement of the by product of DNA oxidation, 8-hydroxydeoxyguanosine (8-OHdG). Furthermore, two groups have now correlated increased sperm oxidative DNA damage with poor blastocyst formation in vitro (Zorn *et al.,* 2003a; Meseguer *et al.,* 2006, 2007). Damaged paternal DNA is recognized to be a significant cause for poor blastocyst development (Seli *et al.,* 2004). Finally, a large prospective study of 225 couples planning their first pregnancy found a strong inverse relationship between seminal 8-OHdG concentration and monthly natural fecundity (Loft *et al.,* 2003).

During natural conception or routine IVF, oxidative damage to the sperm membrane will normally block fertilization, preventing the damaged paternal DNA from creating an embryo. However, during IVF-ICSI this natural barrier to fertilization is lost and sperm containing significantly damaged DNA can still achieve fertilization following microinjection (Zorn *et al.,* 2003a). While many of these embryos will ultimately fail at the blastocyst or early fetal stage, there is the potential for a child to be born with damaged paternal derived DNA. The consequences of this are as yet unknown but it has been suggested to include the initiation of genetic defects and childhood cancer (Aitken and Krausz, 2001; Aitken *et al.,* 2003).

3. OVERVIEW ON SPERM DNA DAMAGE

The formation of mature spermatozoa is a unique process involving a series of meiosis and mitosis, changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins and the final addition of protamines, leading to a highly packaged chromatin (Kumaroo *et al.,* 1975; Goldberg *et al.,* 1977; Poccia, 1986). Mature mammalian spermatozoa contain high percentage of protamines, for example, human and mouse sperm nuclei contain more than 85% and 95% protamines

in their nucleoprotein component, respectively (Gatewood *et al.,* 1987; Bellvé *et al.,* 1988; Debarle *et al.,* 1995). In mice, protamines allow the mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei (Ward and Coffey, 1991). In many mammals, spermatogenesis leads to the production of spermatozoa that appear highly homogeneous in form and function. However, in humans, it is apparent that there are large differences between the form and function of spermatozoa among males and within the ejaculate of an individual. Classically, analyses of the differences in spermatozoa among men have been measured by examining sperm concentration, motility and morphology. Although this analysis gives a broad clinical insight, it does not explain why and where differences originate. For a number of years, many laboratories have concentrated on analysing differences in sperm populations by examining chromatin structure. These studies have shown that the major factor affecting chromatin packaging in ejaculated human spermatozoa appears to be linked to faulty or incomplete protamine deposition during spermiogenesis. In numerous studies, spermatozoa from infertile men were found to exhibit sperm chromatin anomalies related to the deposition of protamines (Balhorn, 1982; Foresta *et al.,* 1992; Belokopytova *et al.,* 1993; de Yebra *et al.,* 1993). These anomalies range from altered ratios of protamine 1 and 2 (Balhorn *et al.,* 1988; Belokopytova *et al.,* 1993) to the complete absence of protamine (de Yebra *et al.,* 1993). During the 1990s, several groups have analysed the sperm nucleus further by examining the integrity of the DNA in mature human spermatozoa (Hughes *et al.,* 1996; Aravindan *et al.,* 1997; Kodama *et al.,* 1997; Aitken *et al.,* 1998; Evenson *et al.,* 1999).

3.1. DNA Packaging in Mammalian Spermatozoa

The chromatin contained in the nuclei of mature mammalian spermatozoa is an extremely compact and stable structure. Sperm DNA must be organized in a specific manner (Fig.6), which differs substantially from that of somatic cells, to achieve this unique condensed state (Poccia, 1986; Ward and Coffey, 1991). This DNA organization not only permits transfer of the very tightly packaged genetic information to the egg, but also ensures that the DNA is delivered in such a physical and chemical form that the developing embryo can access the genetic information. Ward (1997) has proposed a model for sperm DNA packaging on the basis of his work and that of other laboratories. This model depicts a mock assembly of chromosomes starting as long strands of DNA that are gradually packaged at four levels of organization within the mature spermatozoon: (i) chromosomal anchoring by the nuclear annulus, (ii) sperm DNA loop domain organization, (iii) protamine decondensation, and (iv) chromosome organization. This model represents the intricacies of the organization of the sperm nucleus and supports the assumption that anomalies in the DNA would most likely extrapolate to anomalies in overall nuclear organization.

3.2. DNA Damage in Mature Spermatozoa

The integrity of the DNA in mature ejaculated human spermatozoa has been analysed using *in situ* nick translation by sambrook *et al.,* (1989). These experiments were performed by omitting endonuclease treatments, since, in the presence of pre-existing DNA endogenous nicks, the DNA polymerase I, by virtue of its 5'–3' exonucleotic activity can catalyse movement of the nicks along the double helix (Sambrook *et al.,* 1989). The percentage of spermatozoa possessing endogenous DNA nicks in the ejaculates of men have shown a correlation with reduced fertility (Bianchi

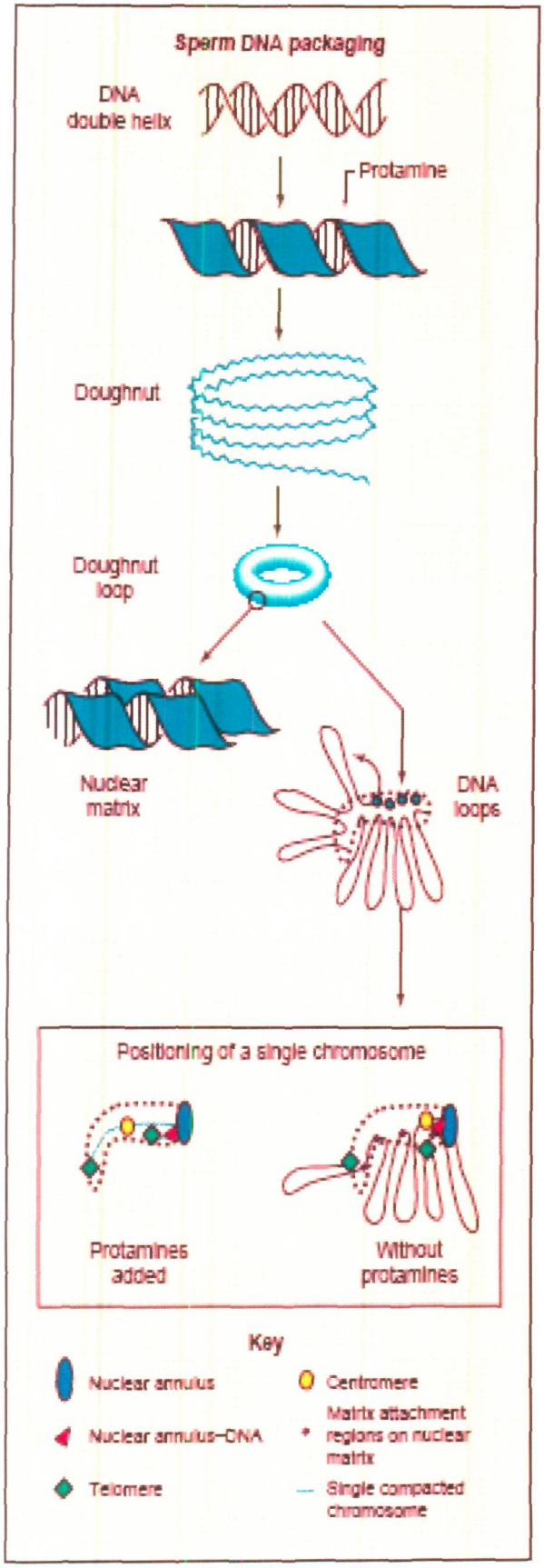


Figure 6. Levels of DNA packaging in sperm chromatin based on the doughnut loop model. Protamine binds to the DNA lengthwise along the double helix. The protamine-bound DNA is coiled into concentric circles. These circles of one loop then collapse into a doughnut into which the neutral DNA protamine complexes are tightly packed. Each doughnut represents one DNA loop domain attached to the sperm nuclear matrix. Single chromosomes are organized with centromeres located in the centre of the nucleus. Each chromosome is attached to the nuclear matrix at intervals of about 40 kb leading to DNA loop domain formation. (Adapted from Ward, 1993, 1997.)

et al., 1993, 1996; Manicardi *et al.,* 1995, 1998; Sakkas *et al.,* 1996). In most cases, the spermatozoa of men with oligoasthenoteratozoospermia display a higher percentage of nicks.

The terminal deoxynucleotidyl transferase (TUNEL) assay has also been used to show the incidence of DNA fragmentation in human spermatozoa Correlation between abnormal sperm chromatin packaging (poorly protaminated spermatozoa) and the presence of DNA strand breaks has been shown (Gorczyca *et al.,* 1993a; Sailer *et al.,* 1995; Manicardi *et al.,* 1998). Sun *et al.,*. (1997), in a cohort of 298 patients, using TUNEL labelling and fluorescence-activated cell sorting. These studies have shown a negative correlation between the percentage of DNA fragmentation and the motility, morphology and concentration of ejaculated spermatozoa. Lopes *et al.,* (1998) have also shown that the percentage of spermatozoa with DNA fragmentation was negatively correlated with fertilization rates obtained with intracytoplasmic sperm injection (ICSI).

The differences in the mode of action between nick translation and the TUNEL technique can be explained when considering that DNA polymerase, being primer- and template dependent, cannot label blunt-ended or 5'-recessed DNA fragments, and that TUNEL, being template-independent, can label all types of fragments at the hydroxylated 3' ends (Gold *et al.,* 1994). However, data reveal that the two techniques cannot distinguish differences in the presence of endogenous DNA damage in human spermatozoa (Manicardi *et al.,* 1998).

The sperm chromatin structure assay (SCSA), which measures the susceptibility of DNA to heat- or acid-induced denaturation *in situ,* is also effective in identifying fertility potential (Evenson *et al.,* 1980). However, accurate measurement of DNA damage in a single spermatozoon has proved difficult. Although techniques such as *in*

situ nick translation and the TUNEL assay can be used to observe a single spermatozoon, there are limitations to the sensitivity of these techniques, and it is possible that many spermatozoa with damaged DNA may escape detection. Other methods for assessing the DNA of a single spermatozoon, such as the Comet assay (single cell gel electrophoresis), are also being established (Hughes *et al.,* 1996; Aravindan *et al.,* 1997). This assay has been recognized in many cell lines to be one of the most sensitive techniques available for measuring DNA strand breaks (Collins *et al.,* 1997). Aravindan *et al.,* (1997) established a significant relationship between the Comet assay for human spermatozoa, the SCSA and TUNEL assays. The Comet assay has been used to examine the effect of various chemicals, such as food mutagens and oestrogenic substances, on spermatozoa *in vitro* (Anderson *et al.,* 1997a,b) and may prove to be one of the more sensitive tests for examining DNA strand breaks in sperm chromatin.

3.3. Origin of DNA damage in mature spermatozoa

In humans, it is clear that the population of spermatozoa in an ejaculate can be highly heterogeneous. Unfortunately, this appears to be more evident in patients whose sperm parameters fall below normal WHO values (WHO, 1992). The positive relationship between poor sperm parameters and DNA damage in spermatozoa points to inherent problems in spermatogenesis in specific patients. Two theories have been proposed to explain the phenomenon of why there are anomalies in the DNA of ejaculated human spermatozoa. The first theory arises from studies performed in animal models and is linked to the unique manner in which mammalian sperm chromatin is packaged. Endogenous nicks in DNA have been shown to be present normally at specific stages of spermiogenesis in rats and mice, and are thought to have a functional significance (McPherson and Longo, 1992, 1993a,b; Sakkas *et al.,*

1995). In the rodent species, endogenous nicks are evident during late spermiogenesis (step 12–13 rat spermatids) but are not observed once chromatin packaging is completed (McPherson and Longo, 1993a; Sakkas *et al.,* 1995). Therefore, the presence of nicks is greatest during the transition from round to elongated spermatids in the testis and occurs before the completion of protamination in maturing rat and mouse spermatozoa (McPherson and Longo, 1992, 1993a,b; Sakkas *et al.,* 1995). In considering the remodelling of chromatin, McPherson and Longo (1992, 1993a,b) postulated that chromatin packaging may necessitate endogenous nuclease activity to create and ligate nicks that facilitate protamination. They proposed that the endogenous nuclease, topoisomerase II (topo II), may play a role in both creating and ligating nicks during spermiogenesis. These nicks are thought to provide relief of torsional stress and to aid chromatin rearrangement during the displacement of histones by protamines (McPherson and Longo, 1992). Chen and Longo (1996) have also shown that changes in DNA topo II expression and localization patterns are consistent with the involvement of topo II in mediating DNA modifications and chromatin changes during rat spermatogenesis. Therefore, the presence of endogenous nicks in ejaculated spermatozoa indicates incomplete maturation during spermiogenesis. This hypothesis is supported by observations that the presence of DNA damage in mature spermatozoa is correlated with poor chromatin packaging due to underprotamination (Gorczyca *et al.,* 1993a; Manicardi *et al.,* 1995; Sailer *et al.,* 1995). The second theory proposes that the presence of endogenous nicks in ejaculated human spermatozoa is characteristic of programmed cell death, as seen in apoptosis of somatic cells, and functional elimination of possibly defective germ cells from the genetic pool (Gorczyca *et al.,* 1993a,b).

Oxidative stress does not simply disrupt the fertilizing capacity of human spermatozoa, it also attacks the integrity of the DNA carried in the sperm nucleus and mitochondria. A variety of techniques have been used to demonstrate the presence of DNA fragmentation in human spermatozoa, including comet, nick translation and sperm chromatin structure assays (Hughes *et al.,* 1996; Evenson *et al.,* 1999; Irvine *et al.,* 2000). DNA fragmentation appears to be inversely correlated with semen quality, particularly sperm count, morphology and motility (Shen *et al.,* 1999; Irvine *et al.,* 2000; Muratori *et al.,* 2000; Shen and Ong, 2000). Moreover, negative correlations have been observed between the stability of DNA in the sperm nucleus and the fertilizing capacity of spermatozoa in vivo and in vitro (Sun *et al.,* 1997; Aitken *et al.,* 1998; Evenson *et al.,* 1999; Host *et al.,* 2000). The ability of the embryo to survive to term also appears to be negatively correlated with the level of DNA fragmentation in the germ line (Host *et al.,* 2000).

That oxidative stress is correlated with DNA fragmentation and this has been demonstrated in many independent studies. Firstly, the DNA in the ejaculates of infertile men is commonly associated with oxidative damage as reflected by measurements of 8-hydroxydeoxyguanosine (8-OHdG) (Kodama *et al.,* 1997; Irvine *et al.,* 2000; Shen and Ong, 2000). Secondly, correlations have been observed between oxygen radical generation and DNA damage in ejaculated spermatozoa (Fig.7) (Barroso *et al.,* 2000; Irvine *et al.,* 2000). Oxidative stress in the male germ line can also be promoted by the presence of transition metals such as iron, copper and nickel that stimulate free radical generation and DNA damage (Liang *et al.,* 1999; Wellejus *et al.,* 2000). Some protection against metal-catalysed DNA damage may be afforded by protamination of sperm chromatin. The N terminus of human protamine P2 contains a heavy metal trap with particular affinity for Ni(II) and Cu(II) (Liang *et*

al., 1999). Therefore, protamines may serve a protective function by sequestering metals capable of promoting the fragmentation of sperm DNA. This function may account in part for the extensive DNA damage observed in poorly packaged spermatozoa in which the protamine–histone transition has been incomplete (Bianchi *et al.*, 1993).

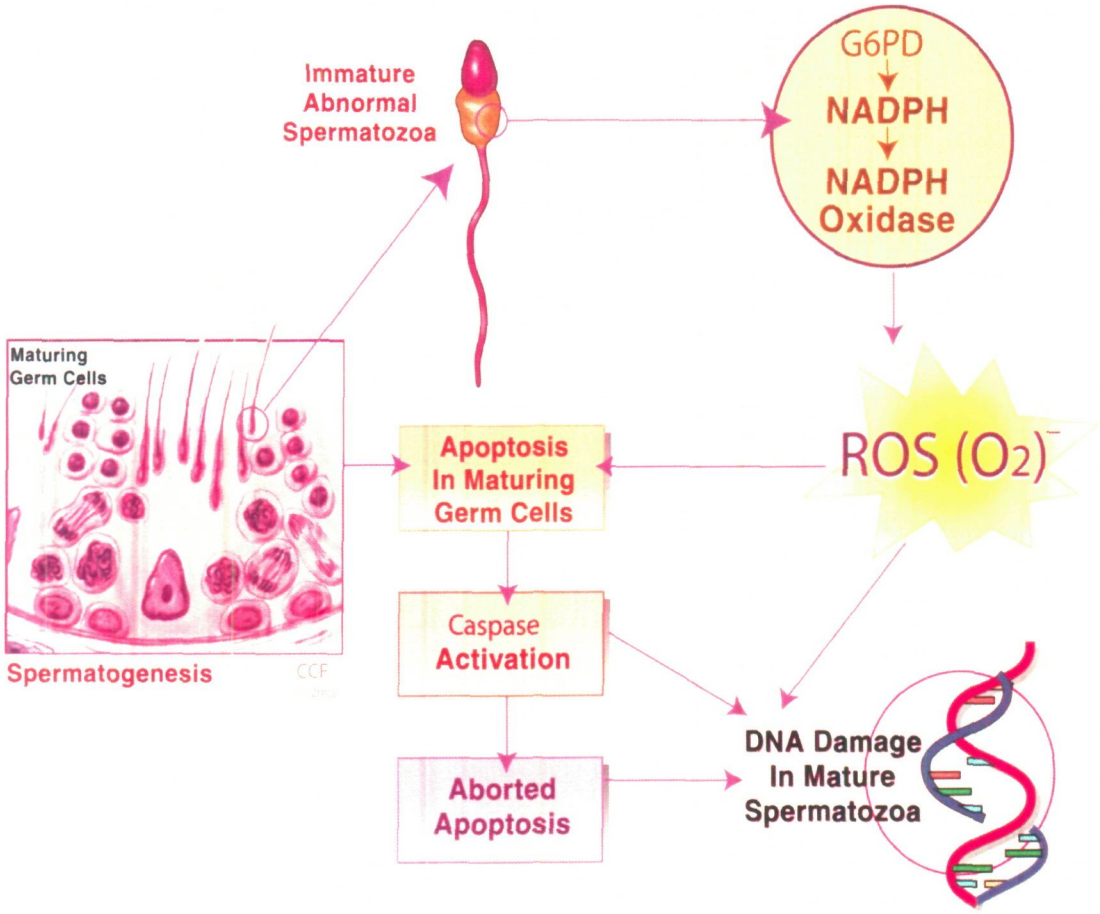


Figure 7. Mechanistic pathway showing sperm DNA damage due to oxidative stress

An alternative aetiology for the DNA nicks seen in the spermatozoa of infertile patients involves an abortive apoptotic pathway mediated by Fas. The induction of apoptosis via the Fas pathway is clearly an important mechanism by which Sertoli cells regulate the number of germ cells, particularly in times of stress (Boekelheide *et al.*, 2000). Accordingly, men exhibiting deficiencies in the semen profile, particularly oligozoospermia, possess a large number of spermatozoa bearing

Fas, prompting the suggestion that these dysfunctional cells are the product of an incomplete apoptotic cascade (Sakkas *et al.,* 1999a). Whether defective apoptosis accounts for a significant proportion of the DNA damage seen in the spermatozoa of infertile men is still an open question. A recent analysis of DNA damage in the germ line did not find ultrastructural evidence for apoptosis in association with DNA damage (Barroso *et al.,* 2000), whereas another study found no correlation between DNA damage and Fas expression (Muratori *et al.,* 2000). Of course, Fas binding and ROS generation are not mutually exclusive phenomena; ROS can induce Fas-mediated signal transduction in some types of cell (Huang *et al.,* 2000), whereas Fas-induced apoptosis appears to be mediated by ROS in other types of cells (Sayers *et al.,* 2000).

Double-stranded DNA breaks also occur naturally in the male germ line both in preparation for recombination and during the process of chromatin packaging (Sakkas *et al.,* 1999b). These physiological strand breaks are normally resolved by the spermatid stage of spermatogenesis. Therefore, it is possible that aberrant recombination– chromatin packaging accounts for unresolved doublestrand breaks in mature human spermatozoa; however, evidence to support this contention is currently lacking.

3.4. Consequences of DNA damage in human spermatozoa

It has been established that there are some anomalies in the DNA of ejaculated spermatozoa. However, the consequences of this DNA damage during fertilization and embryo development are unknown. The increased presence of these anomalies in males with abnormal sperm parameters puts the population of patients being treated by assisted reproductive technologies, in particular ICSI, at the greatest risk. Whether

DNA-damaged spermatozoa can impair the process of fertilization or embryo development is not clear. Studies by Robaire and co-workers have indicated that damage to sperm DNA may be linked to an increase in early embryo death. They showed that treatment of male rats with cyclophosphamide had little effect on the male reproductive system, but resulted in single-strand DNA breaks in the cauda epididymal spermatozoa and altered the decondensation potential of spermatozoa (Qiu *et al.*, 1995a,b). Similar treatment protocols using cyclophosphamide produced an increase in postimplantation loss and malformations (Trasler *et al.*, 1985, 1986, 1987) and were transmissible to the next generation (Hales *et al.*, 1992). In humans, failed fertilized oocytes injected with spermatozoa from patients with a large number of endogenous DNA nicks in their sperm population contain more condensed spermatozoa (Sakkas *et al.*, 1996). This finding indicates that DNAdamaged spermatozoa selected for ICSI may impede the completion or initiation of decondensation, leading to a failure of fertilization. Lopes *et al.*, (1998) have shown that men with a sperm population containing > 25% DNA damage are more likely to experience a fertilization rate of < 20% after ICSI. In addition, in humans, ICSI patients have a lower percentage of embryos that form blastocysts when compared with patients undergoing routine IVF (Shoukir *et al.*, 1998). The use of ICSI has highlighted the risk that spermatozoa containing damaged DNA may participate in the development of an infant. Whether spermatozoa possessing damaged DNA will fail in their project to contribute to a viable offspring at the time of fertilization, embryo development or fetal development is not well understood.

4. ROLE OF SEX HORMONES IN MALE INFERTILITY

The hypothalamus is the integrative center of the reproductive axis and receives messages from both the central nervous system and the testes to regulate the

production and secretion of gonadotropin releasing hormone (GnRH). Neurotransmitters and neuropeptides have both inhibitory and stipulatory influence on the hypothalamus. The hypothalamus releases GnRH in a pulsatile nature which appears to be essential for stimulating the production and release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Fig.8). Interestingly and paradoxically, after the initial stimulation of these gonadotropins, the exposure to constant GnRH results in inhibition of their release. LH and FSH are produced in the anterior pituitary and are secreted episodically in response to the pulsatile release of GnRH. LH and FSH both bind to specific receptors on the Leydig cells and Sertoli cells within the testis. Testosterone, the major secretory product of the testes, is a primary inhibitor of LH secretion in males.

Testosterone may be metabolized in the peripheral tissues to the potent androgen dihydrotestosterone or the potent estrogen estradiol. These androgens and estrogens act independently to modulate LH secretion. The mechanism of feedback control of FSH is regulated by a Sertoli cell product called inhibin. Decreases in spermatogenesis are accompanied by decreased production of inhibin and this reduction in negative feedback is associated with reciprocal elevation of FSH levels. Isolated increased levels of FSH constitute an important, sensitive marker of the state of the germinal epithelium.

The failure of pituitary to secrete FSH and LH will result in disruption of testicular function leading to infertility. Low serum testosterone levels with raised gonadotropin entail damage to leydig cells and seminiferous tubules. Testosterone estradiol and inhibin control the secretion of gonadotropin and also auto regulates its plasma concentration by acting on the hypothalamic-pituitary axis (HPA) to inhibit

LH secretion. Testosterone is secreted by the leydig cells under LH stimulation and is essential for promoting spermatogenesis while FSH has a role in the development of

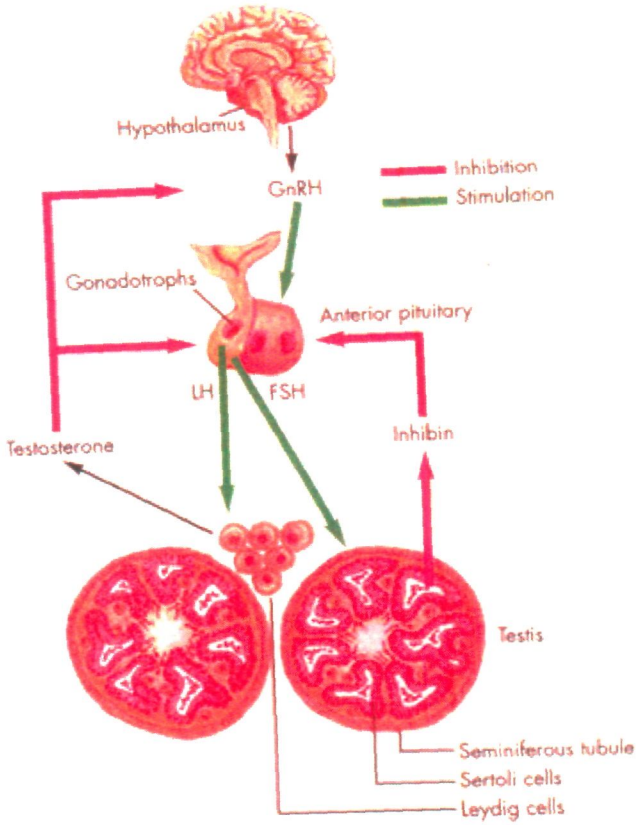


Figure 8. The hormonal regulation of spermatogenesis

the immature testis. The increased FSH level in men with oligozoospermic and asthenozoospermic indicates damage to somniferous tubules (Kati *et al.*, 2006; Matthiesson *et al.*, 2006). A clear understanding of testicular function and related defects may help in counteracting this situation. An azoospermic patient with normal serum testosterone, LH and FSH levels has either retrograde ejaculation or obstructive azoospermia and may need further evaluation with testicular biopsy and exploration to the vas differentia. In contrast, a patient with oligo/azoospermia and elevated circulating FSH but normal LH and testosterone levels has primary germinal tubular failure with no associated leydig cell damage (Islam and Trainer, 1998). A better

understanding of the hormonal requirements of adult spermatogenesis is desirable for the improvement of rational treatments for human infertility.

In the male, elevated concentrations of LH can result from hypergonadotrophic hypogonadism which may be due to various causes such as primary testicular failure, seminiferous tubule dysgenesis, Sertoli cell failure, and anorchia (Franchimont 1973; Marshall, 1975). In sexsually matured adults, low concentrations of LH, FSH and steroids are observed in gonadotrophin deficiency. LH regulates male sexual differentiations, pubertal androgenization, male sexual function, and fertility through the function of leydig cells. Thus, abnormalities or defects in the LH would disrupt the regulatory function of Leydig cells and result in male infertility.

Prolactin secretion from pituitary lactotrophs is under the tonic inhibitory control of dopamine secreted from the hypothalamus, and hyperprolactinaemia arises either from interference with the action of dopamine or from a lactotroph adenoma. Screening of infertile males reveals hyperprolactinaemia in 1.5 – 9% but in the vast majority the hyperprolactinaemia is mild, with normal serum gonadotrophin and testosterone levels, and is unlikely to contribute to the infertility (Jequier *et al.,* 1979; Segal *et al.,* 1976; Carter *et al.,* 1978). Hyperprolactinaemia probably causes hypogonadism by disrupting GnRH release from the hypothalamus (Carter *et al.,* 1978; Thorner and Besser, 1978; Franks *et al.,* 1978). Elevated circulating prolactin concentrations are associated with impotence and decreased libido rather than with spermatogenic failure (Thorner *et al.,* 1977). The impotence may not always be related to testosterone deficiency as replacement does not consistently result in a return of potency, while normalization of serum prolactin does (Franks *et al.,* 1978).

The underlying mechanism for this discrepancy is not understood but prolactin may act centrally to influence potency.

5. INDIAN HERBAL DRUGS

Sexual dysfunction is a common problem with increase in prevalence and etiological factors, including degenerative diseases, increase in injuries and stress associated with industrialized lifestyles. Sexual dysfunction can be treated by both medical and surgical treatment modalities; however, plant-derived and herbal remedies continue to be a popular alternative for men and women seeking to improve their sexual life (Rowland and Tai, 2003). In many countries, different varieties of plants have been used as sexual stimulants in traditional medicine.

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank *et al.,* 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989). This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that "natural" products are harmless. However, the use of these substances is not always authorised by legal authorities dealing with efficacy and safety procedures, and many published papers point to the lack of quality in the production, trade and prescription of phytomedicinal products.

It is estimated that, in 1997, the world market for over the counter phytomedicinal products was US$ 10 billion, with an annual growth of 6.5% (Soldati,

1997). The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries (Vulto and Smet, 1998; OMS, 1991). Eastern countries, such as China and India, have a well-established herbal medicines industry and Latin American countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products, following the example of European countries, such as France and Germany.

Since ancient times, plants have been an exemplary source of medicine. Ayurveda, Unani other Indian literature mention the use of plants in treatment of various human ailments. India has about 45 000 plant species and among them, several thousands have been claimed to possess medicinal properties. Researches conducted in last few decades on plants mentioned in ancient literature or used traditionally for infertility have shown anti-infertility property. In ancient Indian system of medicine, the Ayurvedic and Unani, innumerable plants and plant products e.g. *Tinospora cardifolia* (Guduchi, Menispermaceae), *Asparagus racemosus* (Shatavari, Liliaceae), *Withania somnifera* (Ashwagandha, Solanaceae), *Mucuna pruriens* (Kiwach Papilionaceae), *Orchis latifolia* (Salep, Orchidaceae) etc. are characterized by the qualities of improvement of endurance against stress, general resistance against retardation of the aging process and improving male sexual disorders like psychogenic impotence and unexplained infertility (Ahmad, 1991 and Dhanukar and Hazra, 1995). Seeds, leaves, legumes, roots and tubers of these plant extracts help in some central mechanisms to increase secretion of semen, decrease spermatorrhoea or act as restorative and invigorator tonic aphrodisiac in diseases characterized by weakness or loss of sexual power (Nadkarni, 1986 and Scatezzini and Speroni, 2000).

5.1. *Mucuna pruriens*

Mucuna pruriens (Fabaceae) (Fig 9) is an established herbal drug in the Unani and Ayurvedic system of medicine used for the management of male infertility, nervous disorders and also as an aphrodisiac (Pandey and Chunekar, 1996). Its English name is cowhage and in Hindi it is known as Kinanchh or Kewach. It is an annual herbaceous twining plant, found all over India, especially in the tropics (Chopra *et al.,* 1956). The seeds of this plant are of medicinal importance. They are black and bean-shaped. 80% of the total alcohol extract of the seeds shows the presence of 5-indolic compounds, especially tryptamine and 5-hydroxytryptamine (Pant and Joshi, 1970). Seeds of *M. pruriens* also possess antioxidant, hypoglycemic, lipid lowering and neuroprotective activities (Sharma *et al.,* 1978). Its seeds contain the alkaloids, mucanine, mucunadine, mucanadinine, pruriendine and nicotine, besides ß-sitosterol, glutathione, lecithin, vernolic acid and gallic acid. They posses a number of other bioactive substances, including tryptamine, alkylamines, steroids, flavonoids, coumarins, cardenolides and metals like Mg, Cu, Zn, Fe etc (Vadivel and Janardhan, 2005; Prakash *et al.,* 2001; Misra and Wagner, 2007). It is also rich in fatty content (Table-1)(Panikkar *et al.,* 1987).

The seeds of *M. pruriens,* due to presence of L-3, 4 dihydroxyphenyl alanine (L-DOPA) (Fig. 10) a neurotransmitter precursor, have been used as an effective drug for relief in Parkinson disease and also given as an aphrodisiac (Molly *et al.,* 2006). L-Dopa is an amino acid that converts into dopamine. Dopamine is an essential component of our body and it is required for proper functioning of the brain. The amino acid tyrosine is metabolized into L-dopa and then it converted into dopamine. Without the neurotransmitter dopamine to serve a damping effect on neural

- FAMILY: Fabaceae

  Genus: Mucuna

  Species: pruriens

  Common name: Velvet bean, Kiwach

  Part used: seeds

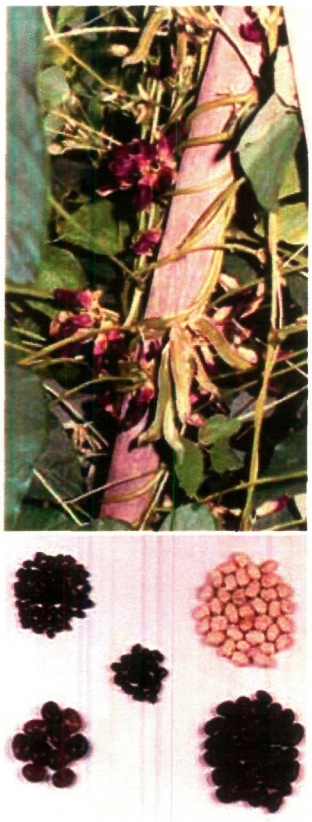


Figure 9. ***Mucuna pruriens*** **(Kiwach)**

TABLE 1

The major biologically active components of *Mucuna pruriens* seeds.

Constituents*	% wt/wt	Metals	mg per 100 g seed flour
L-DOPA	3.6–4.2	Magnesium	174.9–387.6
Alkaloids (mucunine, mucunadine, prurienidine and nicotine)	0.53	Zinc	5.0–10.9
Ascorbic acid	4.78	Iron	10.8–15.0
Total protein (albumins, globulins, prolamins, glutelins) and amines (tryptamine, alkylamine)	20.2–29.3	Copper	0.9–2.2
Total lipids (β-sitosterol, lecithin)	6.3–7.4	Manganese	3.9–4.3
Total dietary fiber	8.7–10.5	Sodium	43.1–150.1
Ash	3.3–5.5	Potassium	778.1–1846
Energy level	1,562–1,597 kJ 100 g^{-1} DM	Phosphorus	325.8–592.0

Note: L-DOPA = L-3,4 dihydroxyphenyl alanine.
Data from references 25–27.
* The seeds also contain flavonoids, coumarins, and cardenolides in traces.

Ahmad. Effect of M. pruriens on infertile men. Fertil Steril 2008.

Table 1. Major biological active components of *Mucuna pruriens* seeds.

transmissions, muscles become tense and tremble. L- Dopa contains natural secretagogues which may support the body's ability to stimulate the natural release of growth hormone. The blood carries the dopamine into the brain, where it naturally increases human growth hormone (HGH) production from the pituitary gland. The increased dopamine levels also optimize the production of other hormones, including testosterone, leading to increased sex drive and improved sexual performance for both men and women, beneficial in stimulating muscle growth, as well as burning fat from fat cells.

Benefits of Mucuna pruriens L Dopa:

Improved sleep (promotes deep sleep)
Reduced bodyfat & decreased cellulite
Decreased weinkles
Improved skin texture & appearance
Increased bone density and reversal of osteoporosis
Increased lean muscle mass
Improved mood and sense of well-being
Enhanced libido & sexual performance
Increased energy levels
Improved cholestorol profile & regeneration of organs (heart, kidney, liver, lungs)
Dramatically strengthed immune system

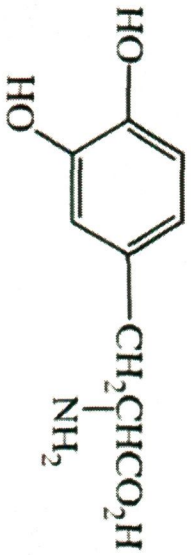


Figure 10. Chemical structure of L-DOPA

Mucuna pruriens has been recognized as an aphrodisiac agent. The plant and its efficacy in treating sexual disorder have been documented in Unani medicine and Ayurveda, but lacks scientific validation. Saksena and Dixit (1987) have reported that the number of spermatozoa increases when the rats were treated with bark extract of *M. pruriens*. Further, it has been reported that the sexual and androgenic activities in adult male rats were sustained while improving the mass of the muscles (Rao and Parakh, 1978; Amin *et al.,* 1996). It is reported to be prophylactic against oligospermia and is useful in increasing sperm count as well as ovulation in the females (Kumar *et al.,* 1994). Alcoholic extracts of the seeds were shown to have

potential antioxidant activity against *in vivo* models of lipid peroxidation induced by stress and alloxan (Tripathi and Upadhyay, 2001).

5.2. Withania somnifera

Family: Solanaceae

Genus: Withania

Species: sominifera

Common name: Ashwagandha, Indian ginseng

Part used: root

Fig 11. Withania sominifera (Ashwagandha)

Withania somnifera Dunal (family, Solanaceae) (Fig 11), known as ashwagandha in Ayurveda, the ancient Hindu system of medicine, has been in use for more than 2500 years. The roots of the plant are categorised as rasayanas, a group of plant-derived drugs that are reputed to promote health and longevity by augmenting defence against diseases, arresting the aging process, revitalising the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and creating a sense of mental well-being (Weiner and Weiner, 1994). The properties ascribed to Ayurvedic rasayanas are remarkably similar to those

said to be present in adaptogens, such as Panax ginseng (PG), which appear to increase nonspecific resistance of the body against diverse stressors and help to promote physical and mental states of the individual (Brekhman and Dardymov, 1969). While reviewing the clinical uses of *W. somnifera* in Ayurveda, Weiner and Weiner (1994) concluded that they include several diseases postulated to be induced by stress.

The chemistry of *W. somnifera* has been extensively studied and over 35 chemical constituents have been identified, extracted, and isolated (Rastogi and Mahrotra, 1998). The biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X) (Fig. 12).

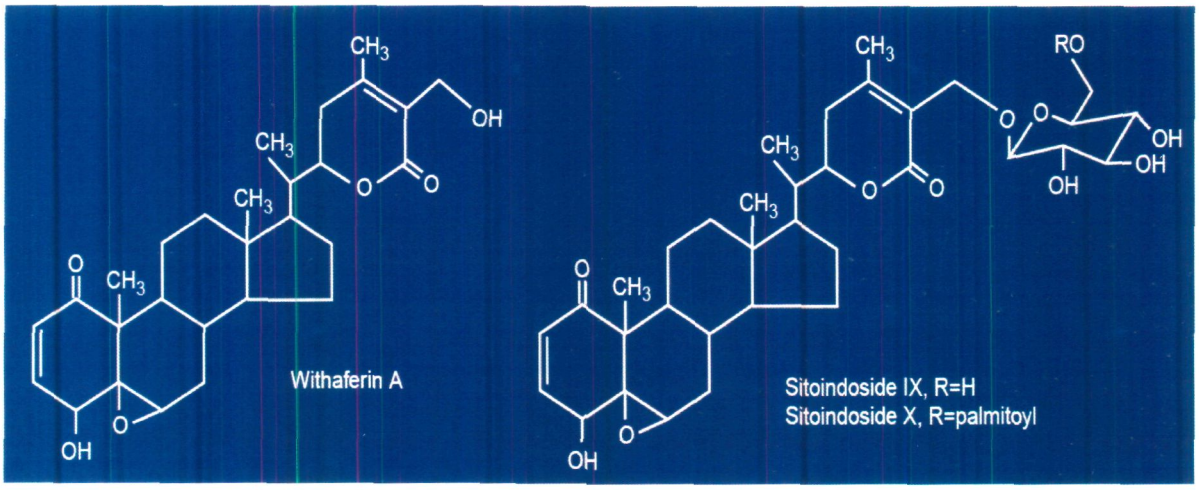


Figure 12 : Chemical structure of withaferin A, and sitoindosides IX and X.

Several earlier investigations have indicated that *W. somnifera* has a profile of activity that is consonant with putative antistress and antioxidant activity. Thus,

W. somnifera, or its major active principles, have anti-inflammatory (Begum and Sadique, 1987; Al-Hindawi *et al.,* 1992), antitumour and radio-sensitizing actions (Devi, 1996) and suppress cyclophosphamide toxicity (Davis and Kuttan, 1998). Likewise, the active principles of *W. somnifera*, comprising sitoindosides VII–X and Withaferin-A, have been shown to have significant antistress activity against acute models of experimental stress (Bhattacharya *et al.,* 1987), immunomodulatory actions (Ghosal *et al.,* 1989), inhibition of cognitive deficits in animal models of Alzheimer's disease (Bhattacharya and Kumar, 1997; Bhattacharya *et al.,* 1995a,b), antioxidant activity in rat brain areas (Bhattacharya *et al.,* 1997a) and anxiolytic–antidepressant action in rats (Bhattacharya *et al.,* 2000a). Similarly, these compounds attenuated iron-overload-induced hepatotoxicity (Bhattacharya *et al.,* 2000b) and streptozotocin induced hyperglycaemia (Bhattacharya *et al.,* 1997b) in rats, which was concomitant with augmented oxidative free radical scavenging activity in the liver and pancreas, respectively. *W. somnifera* was also reported to have significant effects on rat brain neurotransmitter functions (Schliebs *et al.,* 1996). Another study using the same experimental model of chronic stress (CS), reported that there was significant depletion of oxidative free radical scavenging enzymes and an increase in lipid peroxidation in rat frontal cortex and striatum that could be reversed by subchronic administration of WS glycowithanolides (Bhattacharya *et al.,* 2001).

A number of studies (Bhattacharya, 1998; Bhattacharya *et al.,* 2000c; Muruganandam *et al.,* 2002) have shown that chronic unpredictable stress, , can induce glucose intolerance, gastric ulcerations, increase in plasma corticosterone levels, behavioural depression, cognitive deficits, male sexual dysfunction and immunosuppression, associated with increased oxidative stress (Bhattacharya *et al.,* 2001) and significant perturbations in monoamine levels in different rat brain areas

(Bhattacharya *et al.,* 2002). These physiological and biochemical effects of CS are reported to be inhibited by a polyherbal formulation containing *W. somnifera* (Bhattacharya *et al.,* 2002; Muruganandam *et al.,* 2002). Stress has been postulated to be involved in the etiopathogenesis of a diverse variety of diseases, ranging from psychiatric disorders such as depression and anxiety, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions, peptic ulcer, hypertension and ulcerative colitis (Elliott and Eisdorfer, 1982). The benzodiazepine anxiolytics, despite having significant antistress activity against acute models of stress, have not proved effective against CS-induced adverse effects on immunity, behaviour, cognition, peptic ulcer and hypertension (Elliott and Eisdorfer, 1982). *W. somnifera* has been shown to inhibit lipid peroxidation in stress induced animals (Dhuley, 1998). Previous studies have also shown that aqueous extract of this plant elicits changes in pituitary gonadotrophins coupled with an enhancement in epididymal sperm pattern in adult male rats and folliculogenesis in immature female rats (Al-Qarwi *et al., 2000*). *W. somnifera* induced testicular development and spermatogenesis in immature Wistar rats has also been reported and it is said to be by directly affecting the seminiferous tubules (Abdel –Magied *et al.,* 2000).

Objectives of the present study:

In view of above considerations, the present study was designed to investigate the semen, lipid and hormonal profile, oxidative biomarkers and DNA damage of infertile men. Moreover, the protective effects of some Indian medicines on aforementioned parameters were evaluated.

A) Evaluated following parameters:

1) To measure the Sperm count, motility, morphology etc.

2) To asses the levels of lipid profiles i.e. total lipids, cholesterol, triglycerides and phospholipids in seminal plasma of infertile men.

3) To estimate the levels of oxidative biomarkers i.e. lipid peroxides, protein carbonyl groups, total antioxidant capacity in seminal plasma and ROS in spermatozoa of infertile men.

4) To evaluate the activity of seminal antioxidant enzymes namely; superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase.

5) To asses the antioxidant vitamin A, E and C.

6) To estimate the levels of reproductive hormonal profile i.e. LH, FSH, Testosterone and Prolactin in serum of infertile men.

7) To asses the DNA damage in spermatozoa of infertile men.

B) To assess the protection afforded, if any on the aforementioned parameters by the following Indian medicinal plants:

a) *Mucuna pruriens*

b) *Withania somnifera*

Material & Methods

Plant Materials

The seeds of *M. pruriens* and roots of *W. somnifera* were procured from Central Council for Research in Unani Medicine (CCRUM), New Delhi. These were identified and authenticated by Dr. A.K.S. Rawat, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (NBRI). The seeds and roots were dried under shade and made to fine powder using laboratory grinder.

Study Design

The Institutional Review Board and Ethics Committee of Chhatrapati Shahuji Maharaj (CSM) Medical University, Lucknow, approved this study. The study population included normal healthy fertile men (n = 100) and infertile patients (n = 100), aged 20-40 years, recruited from the Outpatient Department of Urology CSM Medical University, Lucknow and infertile male partners of the couples attending the infertility clinic at the Department of Obstetric and Gynaecology, CSM. Medical University, Lucknow. Before enrolment in the study each subject's informed written consent was obtained in response to a fully written and verbal explanation of the nature of study. The potential participants, each with infertility persisting longer than one year, were carefully examined with respect to physical, biochemical and semen parameters. Additionally, medical histories of patients and their female partners were recorded. If infertility in female partner was diagnosed then the patient was excluded from the study. All subjects were instructed to continue normal diet without switching to dietary supplements during the course of treatment.

Subjects:

Study included two groups of the subjects:

1. Control groups (100 normal controls were included in this group)
2. Study groups (this group consist of four sub groups and in each sub-group 25 patients were included)

(A) Control group: Age matched healthy males who had previously initiated at least one pregnancy and exhibited a normal semen profile (>$20x10^6$ spermatozoa/ml; > 40% motility and > 40% normal morphology) constituted the control group.

Exclusion criteria: Subjects who are smokers, and are under stress as also those having diabetes, hypertension, arthritis, malignancies, T.B., HIV positive, infections, endocrinal disorders (hypogonadism, Kallmann's syndrome, hyperprolactinaemia etc.) etc., and on drugs and other conditions known to influence sperm physiology and male fertility, were excluded.

(B) Study group: Infertile subjects from the Outpatient Department of Urology and Male partners of the couples attending to the infertility clinic of the department of Obstetrics and Gynaecology, Queen Mary's Hospital, C.S.M. Medical University, Lucknow whose female partners were apparently normal on clinical examination and on the basis of ultrasound findings as well as also on the basis of the other specialized investigations like diagnostic laparoscopy, hormonal assay and ultrasound guided follicular study, were included in the study group.

Exclusion criteria: Subjects who were smokers, and were under stress as also those having diabetes, hypertension, arthritis, malignancies, T.B., HIV positive, infections, endocrinal disorders (hypogonadism, Kallmann's syndrome, hyperprolactinaemia

etc.) etc., and on drugs and other conditions known to influence sperm physiology and male fertility were excluded.

The study groups were evaluated on the basis of the semen quality into the following categories.

1. Azoospermic: No sperm seen in ejaculated semen specimen or centrifuged pellets of specimens.

2. Oligozoospermic: sperm count less than $20x10^6$/ml, motility more than 40% and more than 40% normal morphology.

3. Asthenozoospermic: Sperm count more than $20x10^6$/ml and motility less than 40% and more than 40% normal morphology.

4. Normozoospermic-infertile: These subjects exhibited normal semen profile i.e. sperm count is more than $20x10^6$/ml and motility is more than 40%, with more than 40% spermatozoa having normal morphology and the female partners had undergone extensive infertility evaluation and none showed a detectable gynaecological abnormality, and infertility is of unknown etiology.

Samples Collection and Preparation

Semen and peripheral blood samples were collected twice, before and after the 3 months of treatment with drugs. Semen samples were collected in sterile plastic containers by masturbation after 3 to 4 days of abstinence and allowed to liquefy for 30 minutes. Semen volume was recorded after liquefaction; an aliquot was taken to assess sperm motility and count. Semen profile was constructed with the procedures described by WHO, 1999. Liquefied semen samples were centrifuged at 1,200 x g for 20 minutes for separation of seminal plasma. The supernatant (seminal plasma) was centrifuged at 10,000 x g for 30 minutes to eliminate all possible contaminating cells.

Seminal plasma was quickly frozen and stored at -20 ^{0}C until the assessment of different biochemical parameters. Peripheral blood samples were drawn between 8 AM and 10 AM and centrifuged at 3,000 x g at 4^{o}C for 10 minutes and serum was aspirated out for hormone assays. Two separate and similar experiments were carried out recruiting same number of subjects in control and study groups to asses the efficacy of *M. pruriens* and *W. somnifera* in infertile men.

Treatment

The dosing schedule was as reported earlier by Singh (1974).

1. *Mucuna pruriens*, (Kiwach): Seed, 5 gm/day (in powder form) was given in a single dose with milk for three months.

2. *Withania somnifera* (Ashvagandha): Roots, 5 gm/day (in powder form) was given in a single dose with milk for three months.

The patients recruited in the study were prescribed these medicines for a period of three months. The dosage of each drug was as per the standard protocol (as specified above) and drugs were administered orally. Semen and blood samples were collected twice, i.e. first before administrating these medicines (0 days) and then after three months (90 days) of treatment with the medicines. The semen and biochemical profiles and DNA damage parameters were assessed. A detailed history of the patients attending the infertility clinic and Outpatient Department of Urology as per the given protocol were taken.

PROFORMA

Name ....Address .... Occupation ....Nature of job/occupation ....

Working hours/day.....Rest day.....Dietary habit: vegetarian/non-vegetarian/omnivorous.....Psychological stress.....Smoking.....Alcohol..... Drugs.....

Infections	
Orchitis	Yes/No
Mumps	Yes/No
Epididymitis	Yes/No
Prostatitis	Yes/No
Urinary tract infection	Yes/No
Sexually transmitted diseases	Yes/No
Gonococcal	Yes/No
Non specific urethritis	Yes/No
HIV (AIDS)	Yes/No
Australia antigen	Yes/No
Chlamydia	Yes/No
Any other	Yes/No
Previous Surgery	
Hernia	Yes/No
Hydrocele	Yes/No
Varicocele	Yes/No
Any other surgery or trauma	Yes/No
Endocrinal Disorder	
a) Diabetes	Yes/No
b) Thyroid	Yes/No
c) Pituitary Dysfunction	Yes/No
Other Disease	
a) Tuberculosis	Yes/No
b) Hypertension	Yes/No
c) Myocardial infarction	Yes/No
d) Renal insufficiency	Yes/No
e) Pancreatic disorder	Yes/No

f) Gastro-intestinal ulceration	Yes/No
g) Any other	Yes/No
Vitamin/Anti-Oxidant Therapy	
a) Vitamin A	Yes/No
b) Vitamin C	Yes/No
c) Vitamin E	Yes/No
d) Captopril	Yes/No
e) Any other	Yes/No

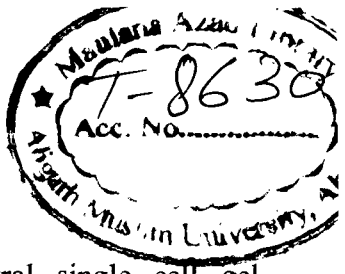
SEMEN ANALYSIS

Physical Appearance

Colour
Volume
Viscosity
Reaction
Liquefaction time

Microscopic Examination

Actively motile
Sluggishly Motile
Poorly Motile
Non-progressively motile
Non-motile
Morphology
Sperm count
Pus cells
Epithelial Cells
Red Blood Cells
Sperm Agglutination
Bacteria
Any other comments



Spermatozoa DNA Damage

DNA damage in spermatozoa was assessed by neutral single cell gel electrophoresis (comet assay) as per the modified method of Singh *et al.,* 2003. Small volume of semen (100–250 µl) was placed in a 1.5-mL polypropylene tube within 30 minutes of collection. Mineral oil was placed over the seminal fluid to prevent further contact with air, and then tubes were kept in ice and incubated in dark room until used. Approximately 10,000 sperm cells were used for making the gel.

For each sample two slides were prepared, fully frosted slides were covered with 100 µl of 0.5% normal melting point agarose (Sigma), a coverslip added and the agarose allowed to solidify. The coverslips were removed and approximately 10,000 spermatozoa in 5 µl of PBS were mixed with 75 µl of 0.5% low melting point agarose (Sigma) and this was used to form the second layer. A final layer of 75 µl of 0.5% low melting point agarose was then added and allowed to solidify. The slides with coverslips removed were then incubated in lysis buffer (containing 1.25 M NaCl, 0.01% sodium *N*-lauroyl sarcosinate, 50 mM tetrasodium ethylenediaminetetraacetic acid, 100 mM Tris, pH 10, 2 mg/mL reduced glutathione and 1 mg/mL of DNase free proteinase K) at 37°C overnight and then placed on a horizontal electrophoretic unit modified to allow electrical input from power supply to both ends of an electrode. The unit was filled with 2 L of 500 mM NaCl, 100 mM Tris HCl, pH 9, and 1 mM EDTA. Slides with microgels were allowed to equilibrate for 20 minutes and electrophoresed for 30 minutes at 24 V (~0.74 V/ cm). The current passing across the sample slides was adjusted to 300 mA by raising or lowering the buffer level. After this, slides for neutralization and DNA precipitation were immersed in freshly prepared 20 mM Tris, pH 7.4 in 50% ethanol with 1 mg/mL of spermine for 15 minutes. This step was

repeated twice with fresh solution. Slides were air-dried. One slide at a time was stained with 80 µL of 20µg/ml ethidium bromide (Sigma-Adrich, USA) and covered with coverslip. All steps were carried out under yellow light to prevent further DNA damage.

Analysis of cells

Each sample was analyses for DNA damage estimation using three aliquots before and after the treatments. A total of 50 cells from each slide were selected randomly and analyzed by image analysis using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. Liverpool, UK. Observations were made at magnifications 400 X using an epifluorescent microscope linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage. Therefore, percentage of head and tail DNA represents the average of total 50 individual cells. The three independent readings for each sample were averaged further to arrive at a final value for each sample. The mean value of all the 25 samples was averaged to get the average for each group (Normozoospermic, Oligozoospermic and Asthenozoospermic). The controls were also evaluated similar as for patients group.

Measurement of Intracellular ROS in spermatozoa

The level of intracellular ROS generation was determined using 2, 7-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Sigma-Adrich, USA), as reported by Wang *et al.*, 1999. Spermatozoa were isolated from swim up process according to WHO, 1999. One ml semen was thoroughly mixed with 2 ml of Ham's F-10 medium and then centrifuged at 800 g for 10 min., the supernatant was discarded. One ml Ham's F-10 was laid down over the pellet gently without

disturbing the pellet and incubated for 30 minutes at 37 °C. Recentrifuged the supernatant at 1200 g for 10 min and suspended in 1 ml of PBS. 4 x 10^5 spermatozoa were added under low-light conditions in medium containing 20 µM working solution of DCFDA. The cells were incubated for 30 min at 37 ^{0}C. After 30 min incubation, medium containing DCFDA was discarded and 200 µl of PBS was added in to the each well and fluorescence intensity was detected using a Multiwell Microplate Reader (Biotek, USA). Excitation and emission wavelength were 485 nm and 528 nm, respectively.

HORMONAL ASSAYS

1. Luteinizing Hormone (LH)

LH levels was measured by ELISA with a kit provided by diagnostic products (CALBIOTECH INC., CA, USA) as per method of Kosasa, 1981.

PRINCIPAL

The essential regents required for an immunoenzymatric assay include high affinity and specific antibodies (enzyme and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-LH antibody.

Test Procedure

Before proceeding with the assay, all the reagents, serum, references and control samples were brought to room temperature. Microplates were formatted for each serum references, control and patient specimen to be assayed in duplicate. Pipette 50 µL of the appropriate serum reference, control or specimen into the assigned well and added 100 µL of LH-Enzyme conjugate solution to all the wells.

Swirl the microplate gently for 20-30 seconds to mix and cover. The plates were incubate for 60 minutes at room temperature. The contents of the microplate were discarded by decantation and dried by blot the plates with absorbent paper. 300 µL of wash buffer was added and briskly shaked out the content of the wells. The wells were rinsed 3 times with wash solution and striked the wells sharply on absorbent paper to remove residual droplets there after added 100 µL of working substrate solution to each well and incubated for 15 minutes at room temperature. Finally 50 µL of stop solution was added to each well and gently mix for 15-20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader within thirty minutes of adding the stop solution. The results were expressed as mIU/mL.

2. Follicle Stimulating Hormone (FSH)

FSH levels was measured by ELISA with a kit provided by diagnostic products (CALBIOTECH INC., CA, USA) as per method of Rose, 1998.

PRINCIPAL

The essential regents required for an immunoenzymatric assay include high affinity and specific antibodies (enzyme and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-FSH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labelled antibody and a serum containing the native antigen, reaction results between the native antigen,

reaction results between the native antigen and the antibodies, without competition or steric hindrance to form a soluble sandwich complex.

Test Procedure

Before proceeding with the assay, all the reagents, serum, references and control samples were brought to room temperature. Microplates were formatted for each serum references, control and patient specimen to be assayed in duplicate. Pipette 50 µL of the appropriate serum reference, control or specimen into the assigned well and added 100 µL of FSH-Enzyme conjugate solution to all the wells. Swirl the microplate gently for 20-30 seconds to mix and cover. The plates were incubated for 60 minutes at room temperature. The contents of the microplate were discarded by decantation and dried by blot the plates with absorbent paper. 300 µL of wash buffer was added and briskly shacked out the content of the wells. The wells were rinsed 3 times with wash solution and stroked the wells sharply on absorbent paper to remove residual droplets there after added 100 µL of TMB-substrate solution to each well and incubated for 15 minutes at room temperature. Finally 50 µL of stop solution was added to each well and gently mix for 15-20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader within thirty minutes of adding the stop solution. The results were expressed as mIU/mL.

3. Testosterone (T)

Testosterone level in serum was measured by ELISA with a kit (DRG) as per modified method of Nardo *et al.,* 2002.

PRINCIPLE OF THE TEST

The DRG Testosterone ELISA kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The mircrotiter wells are coated with an anti directed towards a unique antigenic site on the Testosterone molecule. Endogenous Testosterone of a patient sample competes with a Testosterone horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of Testosterone in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of Testosterone in the patient sample.

Assay Procedure

Before proceeding with the assay, all the reagents, serum, references and control samples were brought to room temperature. Dispense 25 µL of each standard, control and samples with new disposable tips into assigned wells then added 200 µL Enzyme Conjugate solution to all the well. Thoroughly mixed for 30 seconds, it is important to have a complete mixing in this step. The plates were incubated for 60 minutes at room temperature (without covering the plate). There after briskly shacked out the content of the wells. Again the wells were rinsed 3 times with diluted wash solution (400 µL per well). Stroked the wells sharply on absorbent paper to remove residual droplets. Thereafter 200 µL of substrate solution was added to each well and incubated for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µL of stop solution to each well. The OD of reaction mixture was read at 450 nm in a microtiter plate reader within 10 minutes of adding the stop solution. The results were expressed as ng/ml

4. Prolactin (PRL)

Prolactin level in serum was measured by ELISA with a kit (CALBIOTECH INC., CA, USA) as per method of Neidhart, 1996.

PRINCIPAL

The essential regents required for an immunoenzymatric assay include high affinity and specific antibodies (enzyme and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-PRL antibody. Upon mixing monoclonal biotinylated antibody, the enzyme-labelled antibody and a serum containing the native antigen, reaction results between the native antigen, reaction results between the native antigen and the antibodies, without competition to form a soluble sandwich complex.

Test Procedure

Before proceeding with the assay, all the reagents, serum, references and control samples were brought to room temperature. Microplates were formatted for each serum references, control and patient specimen to be assayed in duplicate. Pipette 50 µL of the appropriate serum reference, control or specimen into the assigned well and added 100 µL of PRL-Enzyme conjugate solution to all the wells. Swirl the microplate gently for 20-30 seconds to mix and cover. The plates were incubated for 60 minutes at room temperature. The contents of the microplate were discarded by decantation and dried by blot the plates with absorbent paper. 300 µL of wash buffer was added and briskly shacked out the content of the wells. The wells were rinsed 3 times with wash solution and stroked the wells sharply on absorbent

paper to remove residual droplets there after added 100 µL of TMB-substrate solution to each well and incubated for 15 minutes at room temperature. Finally 50 µL of stop solution was added to each well and gently mix for 15-20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader within thirty minutes of adding the stop solution. The results were expressed as ng/ml

BIOCHEMICAL ASSAYS

1. Lipid peroxides (LPO) (Ohkawa *et al.*, 1979)

Principle: Acetic acid detaches the lipid and protein of the tissues. The protein in the reaction mixture is dissolved by the addition of SDS, Thiobarbiutaric acid (TBA) reacts with LPO, and form the color adduct having absorption maxima at 532 nm.

Reagents:

1. SDS (Sodium dodasyl sulphate)- 8%, 8 gm / 100ml distilled water
2. TBA (Thiobarbiutaric acid)- 0.8%; 0.8 gm / 100 ml distilled water
3. Acetic acid (Glacial)

Procedure: - 0.2 ml of seminal plasma was taken along with 0.2 ml of 8% SDS and mixed well with 1 ml acetic acid. Then added 1.5 ml of TBA and volume was made 4 ml with distilled water. The reaction mixture was heated in a boiling water bath for 1 hour. A pink color was developed along with fine particles, which was centrifuged at 8000 x g for 15 minutes. A clear supernatant was obtained. The OD of this was read at 532nm against reagent blank. The results were expressed as nmol MDA/ml.

2. Protein carbonyl group (Levine and Williams, 1994):

Reagents:

1. 2.5 M HCl
2. 10 mM 2,4 Dinitrophenyl hydrazine (DNPH) in 2.5 M HCl
3. 20% TCA in distilled water
4. 10% TCA in distilled water
5. 6M guanidine HCl solution containing 20 mM potassium phosphate adjusted to pH 2.3 with concentrated HCl.
6. Absolute ethanol: ethyl acetate (1:1 v/v)

Procedure: The experiment was carried out in two setups. In one set (experimental), 5 ml DNPH and 1.5 ml seminal plasma were taken and in the second set (reference) 1.5 ml seminal plasma was added in the 5 ml of 2.5 M HCl instead of DNPH. Tubes were left for 1 hour at room temperature (in dark). The samples were vortexes for 15 min. then 5ml of 20% TCA was added in both sets to a final concentration of TCA by 10% itself. Tubes were kept to ice water for 30 min to get protein precipitated and centrifuged. The protein precipitate was collected, washed with 4 ml of 10% TCA and recovered by centrifugations. Protein pellet was washed 3 times with 4 ml mixture of ethanol: ethyl acetate (1:1 v/v) to remove unreacted DNPH and lipid components. Finally precipitate of experimental and reference were dissolved in 2ml of 6M guanidine HCl and left for 10 min at 37 ^{o}C with general vortex mixing and insoluble material was removed by additional centrifugation. A clear supernatant was obtained. The absorbance of this was measured at 365nm (UV) against guanidine HCl on spectrophotometer. The results were expressed as nmol/mg protein

3. Total antioxidant capacity

A total antioxidant capacity level in seminal plasma was measured by ELISA with an antioxidant assay kit (SIGMA-ALDRICH, USA) as per method of Proteggente *et al.,* 2002.

Principle: The principle of the total antioxidant assay is the estimation of the capacity of test samples to inhibit the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide $^{\cdot}X\text{-}[Fe^{IV}=O]$. This radical oxidizes 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) to produce a radical cation, $ABTS^{\cdot+}$, a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm. The reaction is as follows.

$$HX\text{-}Fe^{III} + H_2O_2 \longrightarrow {}^{\cdot}X\text{-}[Fe^{IV}=O] + H_2O$$

$$ABTS + {}^{\cdot}X\text{-}[Fe^{IV}=O] \longrightarrow ABTS^{\cdot+} + HX\text{-}Fe^{III}$$

In this equation, $HX\text{-}Fe^{III}$ is metmyoglobin and

$^{\cdot}X\text{-}[Fe^{IV}=O]$ is ferryl myoglobin.

Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Trolox™, a water-soluble vitamin E analog, serves as a standard or control antioxidant.

Procedure: 10 µl of seminal plasma and 20 µl of Myoglobin working Solution were added in microtiter wells. After this150 µl of ABTS substrate working solution was added to each well and incubated for 5 minutes at room temperature. The reaction was stopped by adding 100 µl of Stop Solution to each well. Prior to used, the stop solution was warmed at room temperature and mixed until homogeneous. Read

endpoint absorbance at 405 nm using a plate reader on ELISA reader. The results are expressed as mM trolox equivalent.

4. Extraction of lipids (Folch *et al.,* 1956)

Two ml of seminal plasma was mixed with 40 ml mixture of chloroform and methanol (2:1 v/v) solvent mixture. Mixed well and shacked at 40 – 45 °C for 30 minutes. Let the protein precipitated and get it filtered out. Make volume of filtrate to 40 ml with above solvent mixture and added 2 ml normal saline (shake well) and let the layer separate. Discard the upper layer contain water. Washing of lipid extract was repeated again. Finally lipid extract was evaporated at low pressure and temperature until dry. The dry mass of lipid extract was dissolved in 40 ml of hexane and added with 1-2 gm anhydrous Na_2SO_4. Left at room temperature for 2-3 hours, till the extract became clear. Filter the hexane layer in a pre weight glass tube, evaporate to dryness and weight again the tube to calculate the yield of lipids. The results are expressed as mg/dl.

5. Total lipids (Zollenwn and Kish, 1962)

Principle: Vanillin in acidic medium reacts with lipids and formed a rose color as a complex of lipids, vanillin orthophosphate.

Reagents:

Vanillin reagent (13 mM): 197mg vanillin was dissolved in 74.8 ml of diluted orthophosphoric acid (14 ml/litre).

Sufficient amount of triple distilled water was added to a final volume of 100 ml.

Procedure: Mixed 50 µl seminal plasma with 0.5 ml of concentrated H_2SO_4 and heated at 100 °C for 20 minutes to char it. After cooling at room temperature, 3 ml of color reagent was added and allowed to stand for 30 minutes for color development.

Optical density of red (rose) color was read at 540 nm against reagent blank on spectrophotometer. The results are expressed as mg/dl.

6. Total cholesterol (Zlatkis *et al.*, 1953)

Principal:

The Libermann-Bunchard reagent test for quantitative measurement of cholesterol is based on the fact that the sterols with unsaturation in ring A and B reacts with Fe^{3+} dissolved in concentrate H_2SO_4 in the presence of acetic acid to give blue color which immediately change to brown. During the reaction, dehydration, condensation and isomerisation takes place with the formation of thalochromic salt which is brown in color.

Cholesterol + Fe^{3+} - H_2SO_4 $\longrightarrow$ Cholesterol + Fe^{2+} - complex (Brown color)

Reagents:

1. $FeCl_3$-CH_3COOH reagents (Stock solution)- 10% $FeCl_3$ in glacial acetic acid.
2. Glacial acetic acid.
3. Concentrated H_2SO_4.
4. Working reagent: Stock solution diluted to 100 fold into concentrated H_2SO_4.
5. Standard cholesterol solution 1mg/ml in CH_3COOH.

Procedure: Mixed 50 µl seminal plasma with 3 ml acetic acid, added 2 ml working $FeCl_3$ reagent wait 10 minutes for color development. Read color at 575 nm against reagent blank on spectrophotometer. The results are expressed as mg/dl.

7. Phospholipids (Kallner, 1975)

Principal: Inorganic phosphorus preferable reacts with malachite green and in presence of ammonium molybdate. A coordination compound having Pi-dye-molybdate structure with dark blue color is formed. This complex is stabilized in the solution in the presence of high molecular weight polyvinyl alcohol.

Reagents:

Stock reagents:

1. Ammonium molybdate: 28.6 gm dissolved in 500 ml 6N HCl
2. Polyvinyl alcohol (hot water soluble): 11.6 gm dissolved in 500 ml in triple distilled water
3. Malachite green : 812 mg dissolved in 100 ml in triple distilled water.

Working reagents:

Malachite green	: 3 part
Ammonium molybdate	: 1.5 part
Polyvinyl alcohol	: 1.5 part
TDW	: 3 part

This working reagent was prepared on the day of use and allowed to stand at room temperature for 30 minutes or until it turn golden yellow.

Procedure: In hard glass tube 25 µl seminal plasma was mixed with 0.5 ml perchloric acid and heated in sand bath for 1 hour or more till reaction mixture was digested to color less solution. After cooling to room temperature 3 ml working reagents was added and stand for 30 minutes for color development. The OD of this blue color was read at 660 nm on spectrophotometer against reagent blank. The results are expressed as mg/dl.

8. Triglycerides

Triglycerides content in seminal plasma were measured by Spectrophotometric method with a kit (Crest Biosystem, Goa, India) as per method of Fossati and Prencipe, 1982.

Principle: Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol in the presence of glycerol kinase and ATP forms glycerol 3-phosphate which is oxidized with enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinomneimine dye complex. Intensity of the color formed is directly proportional to the amount of triglycerides present in the samples.

Procedure: Mixed 10 µl of seminal plasma with 1 ml of working reagent and incubated at room temperature for 30 minutes. Take the OD at 505 nm against reagent blank. The results are expressed as mg/dl.

9. Catalase *(Aebi 1974):*

Principle: In the UV range H_2O_2 shows a continual increase in absorption with decreasing wave-length and maximum at 240 nm, the decomposition of H_2O_2 can be followed directly by the decease in OD at 240 nm. The €· H_2O_2 at 25 °C was (2.4 ± 0.04) x 10^{-4} M^{-1} cm^{-1}. The decrease in extinction at 240nm per unit time by test sample is used to calculate catalase activity in it.

Reagents:

1. 0.01 M Phosphate buffer pH 7, KH_2PO_4 = 681 mg /dl and Na_2HPO_4 = 1.225g /155 ml were dissolved separately in distilled water and mixed together (Total volume 255 ml).

2. H_2O_2 (0.2 M): Dilute H_2O_2 with buffer to desired concentration.

3. Enzyme source = semen sample

Procedure: 2ml of phosphate buffer and 1 ml. of diluted (0.2M) H_2O_2 were taken in a cuvette, in this 0.02 ml enzyme source was added and mix thoroughly. The decrease in absorbance at 240nm was recorded after every 30 seconds for 3 minutes against reagent blank. One catalase unit is defined as the amount of enzyme required to cause a decrease in optical density by 0.100 of substrate (H_2O_2). Protein content in enzyme source was also determined. The results were expressed as unit/mg protein.

10. Super Oxide Dismutase *(Mc Cord and Fridovich, 1969):*

Principle: NADH in the presence of phenozine methosulpnate (PMS) generate superoxide radical. This oxygen free radical reduces the nitroblue tetrazolium (NBT) and form farmazon having dark blue color. When SOD source is added to above reaction mixture these participate another reaction to neutralize O_2^- in to H_2O_2 and therefore first reaction; the reduction of NBT, slowed down and indicatly give a measure of SOD activity in test sample.

Reagents:

1. Sodium pyrophosphate buffer- 909 mg/dl in TDW pH 8.2

2. Nitroblue Tetrazolium (1.56 mM) – 12.80 mg/10 ml in above buffer

3. NADH (2.34 m mol) – 16.59 mg/10 ml in above buffer

4. Phenozine methosuphate (0.1mM) – 2.8 mg/100 ml TDW

Procedure: The experiment was carried out in two setups. In one setup, 1.1 ml pyrophosphate buffer, 0.2 ml NBT, 0.2 ml PMS, 20µl enzyme source were taken. The second setup received all the above reagents minus the enzyme source. The reaction was started simultaneously in two sets by the addition of 0.2 ml NADH. After an interval of 90 seconds, 0.5 ml glacial acidic acid was added to each tube for checking the reaction, after this same amount of enzyme source was added in reference tubes. The absorbance of both sets tubes was read at 560 nm against reagent blank. Difference between reference and experimental OD of the gives the inhibition of NBT reduction by enzyme source. Protein was also estimated in enzyme source. The unit of SOD enzyme activity was defined as the amount of enzyme required to inhibit the optical density at 560 nm of NBT reduction by 50% in one minute under the assay conditions. The results were expressed as unit/mg protein.

11. Glutathione Reductase *(Hazelton and Lang; 1995)*

Principle: Glutathione reductase catalyses the reduction of oxidized Glutathione by NADPH to reduced Glutathione.

$$GSSG + 2NADPH + 2H^{+} \xrightarrow{GR} 2NADP^{+} + 2GSH$$

Reagents:

1. 0.1 M Tris HCl buffer pH 8.0 – 1.1214 g tris dissolved in TDW and pH was adjust to 8.00 by diluted HCl. The fial volume was make to 100 ml TDW.

2. 1 mM EDTA- 2.2 mg of EDTA was dissolved in 10 ml of TDW.

3. 3 mM GSSG- 18.37 mg dissolved in 10 buffer.

4. 0.1 mM NADPH- 8.34 mg dissolved in 10 ml buffer.

Procedure: The reaction mixture consist of 0.1 ml NADPH, 0.2 ml GSSG, 0.1 ml EDTA, 2.5 ml buffer and 0.1 ml seminal plasma to a total volume of 3.0 ml. The reaction was initiated by the addition of seminal plasma. Oxidation of NADPH was followed at 340 nm. Blank reaction was also run simultaneously. The decrease in absorbance at 340 nm is followed at 30 second intervals. Protein content in the enzyme source was also determined. Enzyme unit was defined as nmole of NADPH oxidized per minute per mg protein. The results were expressed as unit/min/mg protein.

12. Glutathione Peroxidase *(Pagila and Valentine; 1967)*

Principle: During conversion of hydroperoxide radical into non reactive hydroperoxides, Glutathione Peroxidase utililize reduced Glutathione as a cofactor. In this pathway the amount of GSH utilized is a measure of enzyme activity, GSH is converted in oxidized glutathione (GSSG).

Reagents:

1. 0.1 M Phosphate buffer pH-7.0 = 681 mg /dl and Na_2HPO_4 = 1.225g /155 ml were dissolved separately in distilled water and mixed together (Total volume 255 ml).
2. 10 mM Sodium azide- dissolved 6.501 mg in 10 ml buffer
3. 2 mM Glutathione reduced (GSH) - dissolved 6.15 mg in 10 ml buffer
4. 10% TCA
5. 3 mM Na_2HPO_4 - 77.4 mg dissolved in 10 ml buffer
6. 0.1 M 5,5'-Dithio-bis(2-nitrobenzoic acid (DTNB)- dissolved 396 mg in 10 ml buffer

7. 2.5 mM Hydrogen peroxide

Procedure: An incubation mixture containing of 0.4 ml buffer, 0.2 ml of GSH, 0.2 ml EDTA, 0.2 ml Sodium azide and 0.2 ml hydrogen peroxide was pre-incubated at 37 °C for 10 min. 0.1 ml of seminal plasma was added and incubated at 37°C for 10 min. The reaction was terminated by the addition of the 0.1 ml of 10% TCA. Supernatant was taken and 3 ml of phosphate buffer and 1 ml of DTNB were added. The color developed was read immediately at 412 nm in a spectrophotometer. Protein was also estimated in enzyme source. GPx enzyme activity was expressed as μg GSH oxidized per mg protein. The results were expressed as unit/min/mg protein.

13. Protein *(Lowry et. al., 1951):*

Principle: It is a most commonly used method for determination of protein in cell free extracts, because of its high sensitivity and quantities as low as 20 μg of protein concentration be measured. The peptide bond in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue color complex. In addition tyrosine and tryptophan residues of protein reacts with phosphotungstate components of folin reagent to give blue product which contribute toward enhancing the sensitivity of this method.

Reagents:

1. Alkaline Na_2CO_3 Reagent: A

   8 g of Na_2CO_3 dissolved in 0.1 N NaOH to final volume of 100 ml.

2. Copper sulphate Reagent: B

   Prepare 0.5% CuSO4 in 1% Na – K tarterate solution in TDW.

3. Alkaline copper sulphate Reagent: C

Add (1:1 v/v) reagent A and reagent B prepare freshly when used.

4. Folin's Reagent: Dilute the reagent with equal volume of TDW just before use.

Protein Preparation from Semen Sample:

0.1 ml seminal sample was mixed with 0.9 ml normal saline, to a total volume of 1ml. 0.2 ml diluted semen sample was taken and mixed with 0.8 TDW and 1 ml TCA (10%). The tubes were kept at 4 °C overnight. On next day the tubes were centrifuged it at 4000 g for 15 minute. The sediments containing protein was dissolved in 2 ml of 0.1 N NaOH.

Procedure: 0.1 ml protein solution from semen sample was mixed 5 ml reagent C and incubated it at 37 °C for 15 minute. After this 0.5 ml of diluted Folin's reagent was added and further incubated at 37 °C for 30 minute for color development. The blue color was read at 660 nm against reagent blank. Appropriate standard (100 µg) of Bovine serum albumin dissolved in 0.1 N NaOH (1mg/ml) was also run simultaneously.

14. Ascorbic Acid *(Beutler et al., 1988):*

Reagents:

1) 0.2 M citrate phosphate buffer pH 5.6- Dissolved 806.82 mg of citric acid in 25 ml TDW. Separately dissolved 2.064 g Na_2HPO_4 in 50 ml TDW. Mix both solution and make to a final volume of 200 ml by adding required amount of TDW.

2) 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (3.62 mM) - 30 mg / 20 ml

3) Phenozine methosuphate (0.1 mM) – 2.8 mg/100 ml TDW

4) Ascorbic acid 15 mg/10ml in phosphate buffer.

5) Acetic acid glacial

Procedure: The experiment was carried out in two setups. The experiment set required 2 ml of 0.2 M citrate phosphate buffer ph 5.6, 0.2 ml PMS, 0.2 ml MTT and 0.1 ml sample. The second set received all the above reagents added with 0.1 ml standard of ascorbic acid (15 µg) instead of test sample. Both the sets including reagent blank were at 37 °C for 15 minutes. After this the reaction in all the tubes were stopped by adding 0.5 ml CH_3COOH. The optical density of this colour developed was read at 578 nm on spectrophotometer. The results were expressed as mg/dl.

15. Determination of Vitamin A and E: Vitamin A and E were measured by HPLC as per the modified method of Omu *et. al.*, 1999.

Reagents:

1) Solvents : Methanol (95%) : HPLC grade

2) Stocks of 800 mg/l of Trans retinyl acetate, 946 mg/L of α- tocopherol acetate and 420 mg/ml of α- tocopherol 286 mg/l of retinal were used as internal standards and standards, respectively.

Procedure: 200 µl of α-tocopherol acetate (20nmol) and 200 µl of retinol acetate (20nmol) were pepitted into in eppendorf tube. Into this 400 µl of seminal plasma was added and vortex mixed. The mixture was added with 400 µl of hexane (HPLC grade) and centrifuged at 6000 g for 5 min. This separated in three distinct layers, the upper layer containing hexane extract of vitamin A and E were aspirated out in a glass tube, dried under nitrogen stream and finally dissolved in 200 µl of methanol (HPLC

grade), 50 µl of this preparation was injected into HPLC fitted with reverse phase C-18 stainless steel column. The vitamins were eluted with methanol (95%) at the flow rate of 1.5ml/min for 15 minute. The peak height and the curve area of vitamin A and E as well as their acetates were measured to calculate the amount of these vitamins in seminal plasma at UV detector of 292 nm filters. The results of vitamin A and E were expressed as µg and mg.

Statistical Analysis

Data for the cases (pre-treatment) was compared with the control samples to assess the correlation between infertility and various parameters measured in the study. The five independent groups; control, pre-treated normozoospermic, pre-treated oligozoospermic, and pre-treated asthenozoospermic, pre-treated azozoospermic were compared by one-way analysis of variance followed by Dunnett's test.

In the second part of data analysis pre-treatment groups were compared with post-treatment to asses the impact of the treatment. A paired *t* test was used to analyze the significance of mean difference between pre- and post-treatment infertile groups. All hypothesis testing was two tailed. The results were expressed as mean ± SD, and $P<.05$ was considered statistically significant. The statistical analyses were carried out with commercial software (INSTAT 3.0; GraphPad Software, San Diego, CA).

Results

MUCUNA PRURIENS

Semen Profile:

The semen profiles of the fertile (control) group and the pre- and post-*M. pruriens* treated infertile groups are shown in Table 2.

Semen Profile Associated With Male Infertility

In normal healthy fertile men (control groups), sperm concentration ($x10^6$) was 86.80 ± 13.31 /ml, sperm count ($x10^6$) per ejaculate was 232.66 ± 43.51, motility was 77.30 ± 9.47% and semen volume was 2.93± 0.40 ml. It was found that in infertile normozoospermic, oligozoospermic and asthenozoospermic men, there was a decline in their sperm concentration by 35, 89 and 46% and sperm per ejaculate by 46, 92 and 59%, motility by 21, 23 and 80%, following decrease in semen volume by 30, 31 and 20%, respectively. Moreover, the semen volume was found decreased in azoospermic men by 18% as compared with control.

Treatment Improve Semen Profile

Treatment of the above mentioned infertile men with *Mucuna pruriens* seed powder (5gm/day) for three months showed significant reversal of above semen parameters. Our results showed that following treatment the sperm concentration of normozoospermic, oligozoospermic and asthenozoospermic infertile men were increased by 64, 496, and 47%, respectively. Similarly sperms per ejaculate were also found increased by 97, 458 and 66%, as compared with their pre-treatment parameters. Motility of spermatozoa of normozoospermic, oligozoospermic and asthenozoospermic infertile men was found increased by 21, 17 and 54%, and semen volume by 37, 15 and 2 %, respectively as compared with their pre treatment values. Whereas treatment didn't recovered the level of sperm concentration in azoospermic men.

TABLE NO. 2

Effect of *Mucuna pruriens* on semen profile of infertile men

Group	*Treatments*	*Semen volume (mL)*	*Sperm concentration (1×10^6 / mL)*	*Sperm count ($1x10^6$) per ejaculate*	*Motility (%)*
Control (n =100)		2.93± 0.40	86.80 13.31	232.66 ± 43.51	77.30 9.47
Normozoospermic (n = 25)	Pre Treatment	2.04±0.36 a**	55.78±12.19 a**	124.72 ± 32.67 a**	60.83±9.17 a**
	Post Treatment	2.80±0.33 b**	91.71±6.83 b**	244.33 ± 44.04 b**	73.75±6.95 b**
Oligozoospermic (n = 25)	Pre Treatment	2.0±0.30 a**	8.77±1.40 a***	19.01 ± 7.45 a***	58.75±8.50 a**
	Post Treatment	2.31±0.28 b*	52.30±6.23 b***	106.28 ± 27.45 b***	68.96±7.22 b*
Asthenozoospermic (n = 25)	Pre Treatment	2.20 ± 0.31 a**	46.48±6.57 a**	94.70 ± 18.21 a**	15.13±2.44 a**
	Post Treatment	2.52 ± 0.32 b*	68.5±4.34 b**	156.06 ± 19.17 b**	23.25±4.40 b**
Azozoospermic (n = 25)	PreTreatment	2.39±0.66 a*	Nil	Nil	NA
	PostTtreatment	2.54±0.61 NS	Nil	Nil	NA

a* P<.05, a** P<.01, and a*** P<.001 as compared to control groups (Dunnett test).
b* P<.05, b** P<.01, b*** P<.001, as compared to pretreatment groups (paired t test)
NS = Non significant, NA = Not applicable

Status of Lipid Profile in Seminal Plasma of Infertile Men

Mean levels of lipid profile of infertile subjects and controls are listed in Table 3.

The levels of total lipids, cholesterol, triglycerides, and phospholipids in the seminal plasma of fertile men were 376.08 ± 37.23 mg/dL, 59.49 ± 7.25 mg/dL, 62.97 ± 6.94 mg/dL, and 182.63 ± 20.48 mg/dL, respectively. These levels were significantly reduced in all groups of infertile men when compared with controls. The level of total lipids decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 26, 31, 37 and 35%, and cholesterol by 21, 13, 16 and 25%, respectively. Similarly triglycerides level decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 15, 35, 33 and 28%, and phospholipids level by 21, 31, 25 and 34%, respectively.

Treatment Restored the Levels of Lipid Profile In Seminal Plasma

Treatment with *M. pruriens* recovered the level of total lipids in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 28, 32, 27 and 17%, and cholesterol levels by 22, 8, 8 and 11%, respectively, when compared with pre treatment values. Similarly treatment also recovered the level of triglycerides in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 12, 35, 21 and 15%, and phospholipids by 21, 31, 25 and 34%, respectively.

TABLE NO. 3

Effect of *Mucuna pruriens* on seminal lipid profile of infertile men

Group	*Treatments*	*Total lipids (mg/dL)*	*Cholesterol (mg/dL)*	*Triglycerides (mg/dL)*	*Phospholipids (mg/dL)*
Control (n =100)		376.08 ± 37.23	59.49 ± 7.25	62.97 ± 6.94	182.63 ±20.48
Normozoospermic (n = 25)	Pre Treatment[a]	278.08 ± 28.43	46.85 ± 5.80	53.31 ± 6.84	144.91 ± 16.59
	Post Treatment[b]	357.21 ± 29.01	56.01 ± 4.65	59.62 ± 8.43	169.44 ± 16.88
Oligozoospermic (n = 25)	Pre Treatment[a]	261.3 ± 31.1	51.5 ± 8.44	40.83 ± 6.75	126.81 ± 18.80
	Post Treatment[b]	345.8 ± 29.13	55.91 ± 7.48	55.29 ± 8.54	158.16 ± 19.64
Asthenozoospermic (n = 25)	Pre Treatment[a]	237.13 ± 35.2	50.15 ± 7.24	42.45 ± 7.54	136.31 ± 15.39
	Post Treatment[b]	302.26 ± 41.38	54.31 ± 6.12	51.23 ± 7.86	161.13 ± 17.41
Azozoospermic (n = 25)	Pre Treatment[a]	246 ± 32.4	44.37 ± 6.28	45.39 ± 6.97	121.31 ± 15.43
	Post Treatment[b]	289 ± 36.63	49.21 ± 5.62	52.36 ± 5.83	150.43 ± 16.74

[a] $P < .01$ for all parameters compared with control groups (Dunnett test).
[b] $P < .01$ for all parameters compared with pretreatment groups (paired t test).

Oxidative Biomarkers and Male Infertility

It was observed that the level of lipid peroxides (LPO) in seminal plasma of control healthy fertile men was 1.86±0.12 nmol MDA/ml, however, seminal plasma LPO levels in infertile normozoospermic, oligozoospermic and asthenozoospermic men, were increased by 94, 34 and 77%, respectively, while it was found decreased in azoospermic men by 23% (fig. 13). The level of protein carbonyls in the seminal plasma of fertile men was 1.96±0.54 nmol/mg protein. This parameter was found increased in the seminal plasma of normozoospermic, oligozoospermic and asthenozoospermic infertile men by 79, 110 and 50% respectively, following decrease in azoospermics by 39% (fig. 14). The infertile men had significantly high mean percentage of ROS in the ejaculated spermatozoa samples than the control samples and increment was found in normozoospermic, oligozoospermic and asthenozoospermic, infertile men by 164, 195 and 178%, respectively (fig. 15). The level of total antioxidant capacity (TAC) in the seminal plasma of fertile men was 1.78±0.10 nM trolox equivalent. TAC was found decrease in infertile groups of normozoospermic men by 22%, in oligozoospermic men by 39%, asthenozoospermic men by 49% and azoospermic men by 42% (fig. 16)

Treatment Reduced the Levels of Oxidative Biomarkers

After treatment with *M. pruriens* for three months the levels of LPO in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic infertile men, were decreased by 40, 23, 33 and 13%, respectively, as compared with pre treatment parameters (fig. 13). Similarly the levels of protein carbonyls in seminal

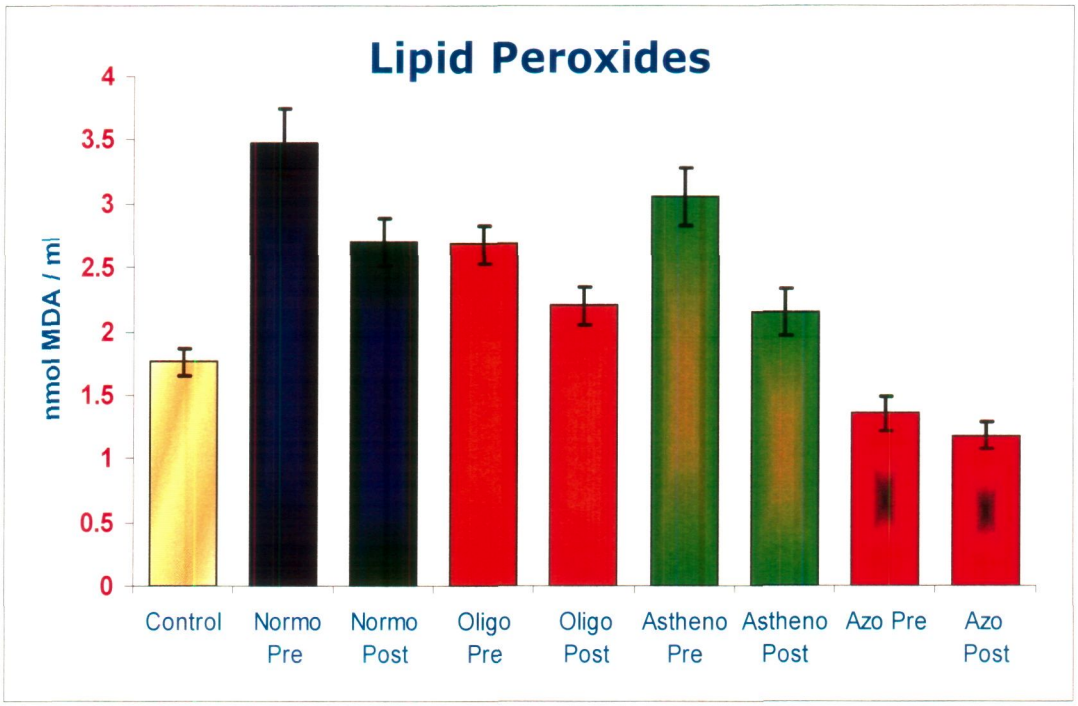


Figure 13: Effect of *M. pruriens* on Lipid peroxide levels in seminal plasma of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

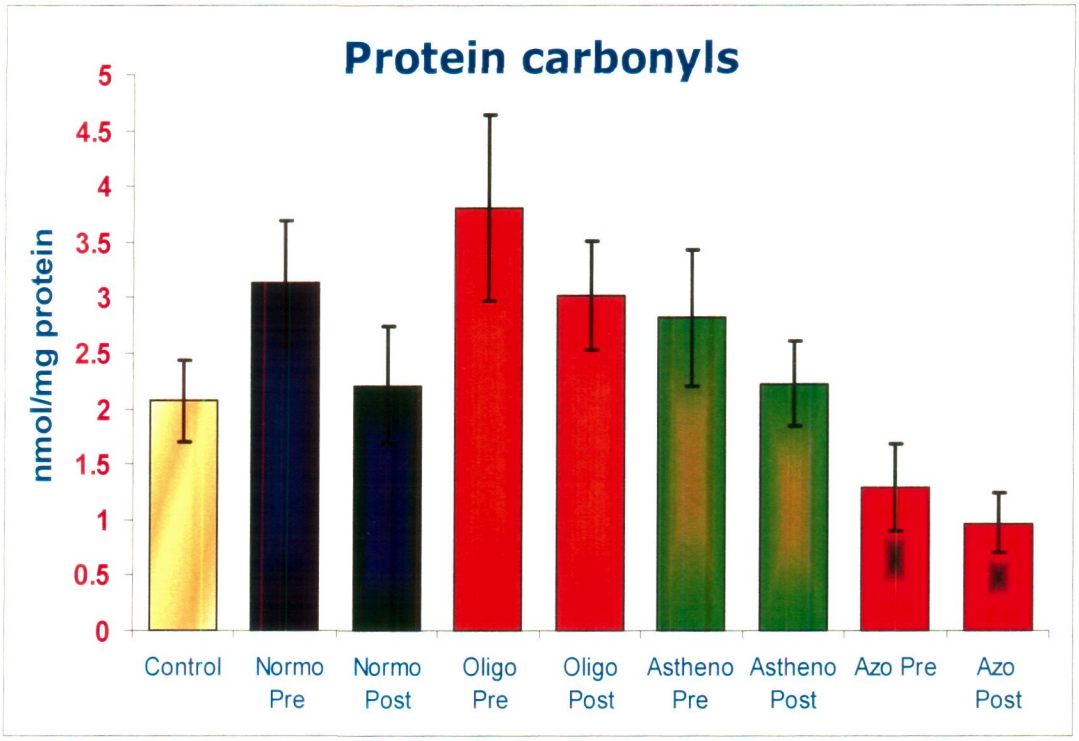


Figure 14: Effect of *M. pruriens* on Protein carbonyl levels in seminal plasma of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

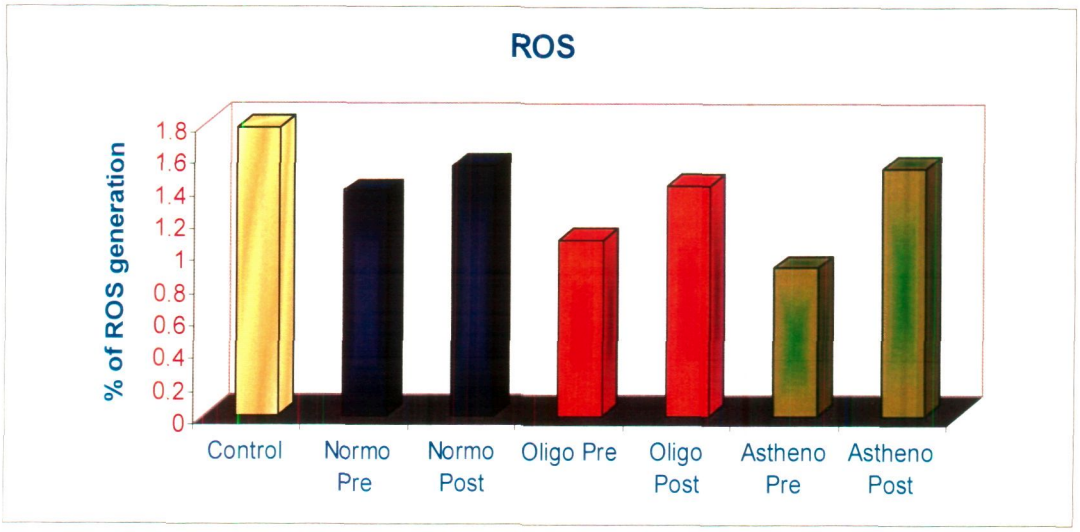


Figure 15: Effect of *M. pruriens* on levels of ROS in spermatozoa of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

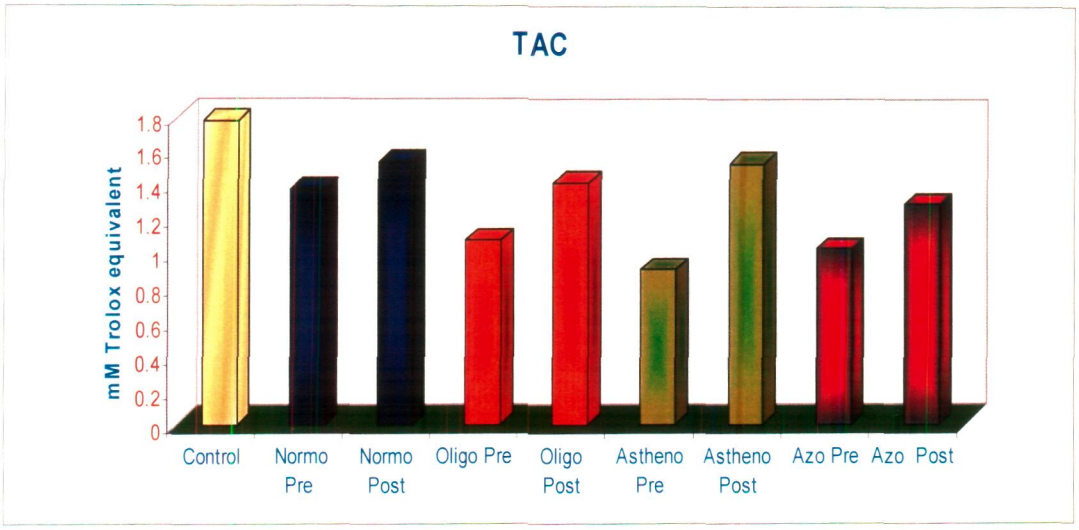


Figure 16: Effect of *M. pruriens* on of seminal plasma TAC levels of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic infertile men were reversed by 44, 30, 29 and 22%, respectively (fig. 14). The mean percentage of ROS in the ejaculated spermatozoa was also significantly decreased upon treatment as compared with their pre treatment values and the decrease was found in normozoospermic, oligozoospermic and asthenozoospermic infertile men by 45, 45 and 39%, respectively (fig. 15). Treatment with *M. pruriens* increases the levels of TAC in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 12, 31, 68 and 25%, respectively (fig. 16).

Status of Antioxidant Enzymes in Seminal Plasma of Infertile Men

The antioxidant enzymes of the fertile (control) group and the pre- and post-*M. pruriens* treated infertile groups are depicted in Table 4.

This was observed that superoxide dismutase (SOD) activity in seminal plasma of healthy fertile controls was 8.24±0.64 unit/mg protein. However, this enzyme activity was found suppressed in infertile groups of normozoospermic men by 22%, in oligozoospermic men by 36%, asthenozoospermic men by 32% and azoospermic men by 40%. Similarly the activity of catalase in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was also found suppressed by 12, 9, 34 and 29%, respectively, when compared with that of fertile control group (9.58±1.39 unit/mg protein).

The glutathione reductase (GR) and glutathione peroxidase (GPx) activity in seminal plasma of fertile men was 15.67± 3.74 U/min/mg protein and 8.45±2.23 U/min/mg protein respectively. However, enzyme activity was found suppressed in infertile

TABLE NO. 4

Effect of *Mucuna pruriens* on the levels of antioxidant enzymes in seminal plasma of infertile men

Group	*Treatments*	*SOD (U/mg protein)*	*Catalase (U/mg protein)*	*GR U /min/mg protein*	*GPx U/min/mg protein*
Control (n = 100)		8.24±0.64	9.58±1.39	15.67±3.74	8.45±2.23
Normozoospermic (n = 25)	Pre Treatment	6.38±0.67^{a*}	8.43±0.53 ^{a*}	13.92±2.78 a*	7.34±1.78 a*
	Post Treatment^{b}	7.57±0.37 ^{b*}	9.42±0.79 b*	15.36±2.52 b*	8.40±1.56 b*
Oligozoospermic (n = 25)	Pre Treatment	5.21±0.61^{a**}	8.69±0.68 ^{a*}	13.74±2.78 ^{a*}	7.58±1.93 ^{a*}
	Post Treatment^{b}	7.35±0.69^{b**}	9.37±0.96 b*	15.23±2.24 b*	8.37±1.78 b*
Asthenozoospermic (n = 25)	Pre Treatment	5.57±0.79 ^{a**}	6.32±0.70 ^{a**}	13.24±3.60 ^{a*}	7.04±1.55 ^{a*}
	Post Treatment^{b}	6.98±0.54 b*	7.10±0.90 b*	14.41±2.77 b*	7.98±1.54 b*
Azozoospermic (n = 25)		4.91±0.65 ^{a**}	6.72±0.63 ^{a**}	12.70±2.72 ^{a**}	6.90±1.55 ^{a*}
		6.31±0.65 b*	6.89±0.48 ^{NS}	13.49±2.44b*	7.32±1.54 ^{NS}

a* P<.05, a** P<.01 as compared to control groups (Dunnett test).
b* P<.05, b** P<.01 as compared to pretreatment groups (paired t test).
NS = Non significant.

normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 11, 12, 15 and 18% and 11, 10, 13 and 18 %, respectively (Table 4).

The Activity of Antioxidant Enzymes Were Recovered Upon Treatment

Treatment with *M. pruriens* recovered SOD activity in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 19, 41, 25 and 28%, and catalase activity by 11, 7, 12 and 2%, respectively, as compared with pre treatment values. Similarly treatment also recovered GR activity in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic by 10, 11, 9 and 8%, and GPx activity by 11, 10, 13 and 6%, respectively. The catalase and GPx activities were not found recovered significantly in azoospermic men.

Male Infertility and the Levels of Antioxidant Vitamins in seminal plasma

The levels of vitamin A in seminal plasma of control fertile group was 27.81± 4.28 µg/dl which was found decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 32, 36, 42 and 54%, respectively (fig. 17). Furthermore, the level of vitamin E in seminal plasma of infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was found decreased by 17, 29, 37 and 54%, as compared to that of control fertile men (0.140±0.012 mg/dl) (fig. 18). The level of vitamin C in seminal plasma of control groups was 5.85± 0.73 mg/dl which was also found decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 23, 9, 12 and 16%, respectively (fig. 19).

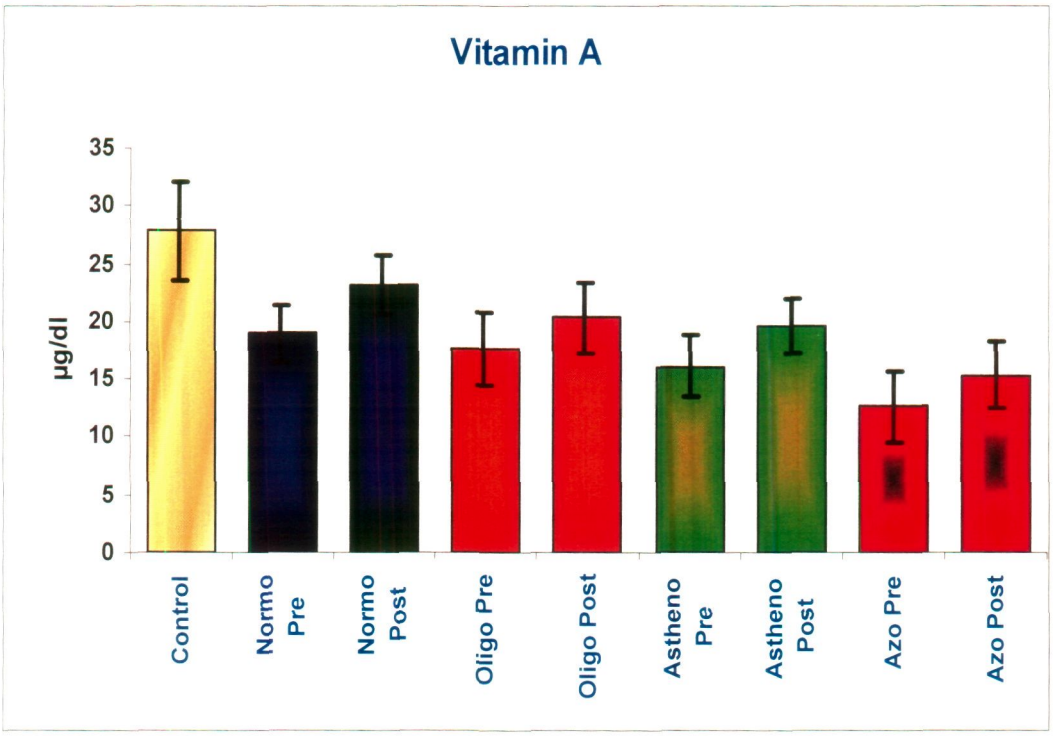


Figure 17: Effect of *M. pruriens* on Vitamin A levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

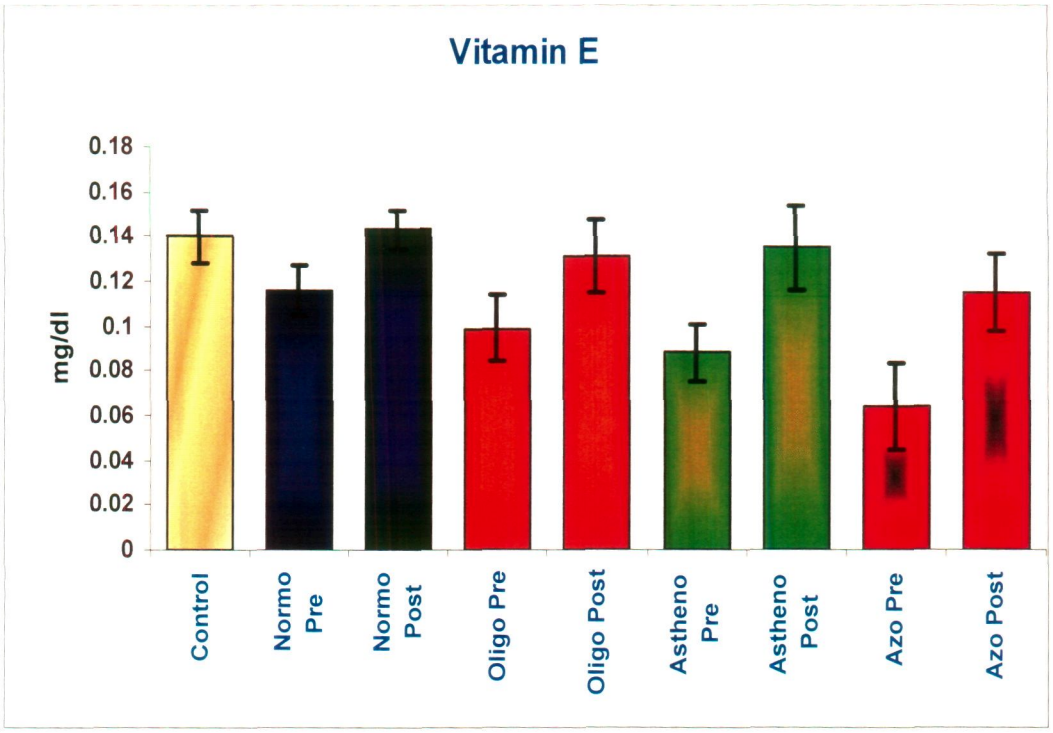


Figure 18: Effect of *M. pruriens* on Vitamin E levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

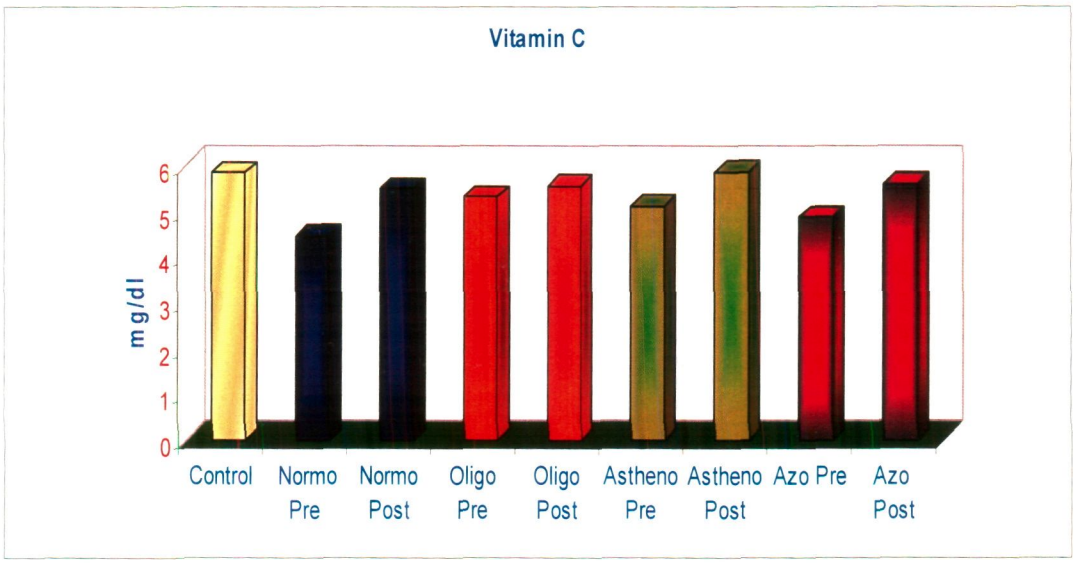


Figure 19: Effect of *M. pruriens* on Vitamin C levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

Treatment Recovered the Levels of Antioxidant Vitamin

Treatment with *M. pruriens* of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men showed recovery in the seminal plasma levels of vitamin A by 22, 15, 21 and 20% respectively (fig. 17). The levels of vitamin E in seminal plasma were found reversed by 23, 32, 53 and 79%, respectively as compared with pre treated values (fig. 18). Similarly, treatment also recovered the level of vitamin C in seminal plasma by 23, 8, 14 and 14%, when compared with pre treatment values (fig. 19).

Hormonal Profile Associated With Male Infertility

Mean basal hormone levels of infertile subjects and controls are listed in Table 5.

Luteinizing hormone (LH) and Testosterone (T)

The serum LH concentration in control group was 7.85 ± 1.17 mIU/mL. This parameter was found significantly low in all infertile men except those who were azoospermic. LH levels were found decreased in infertile normozoospermic, oligozoospermic and asthenozoospermic men by 19, 48 and 50%, respectively, and increased in azoospermic men by about 5%. Furthermore, the level of Serum T of infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was found decreased by 16, 47, 36and 41%, respectively, as compared with that of control fertile men (7.00±0.67 ng/ml).

Follicle-stimulating hormone (FSH) and Prolactin (PRL)

FSH and PRL levels in the serum of fertile control men were 5.86 ± 0.68 mIU/mL and 6.75 ± 0.71 ng/mL, respectively. These parameters were significantly elevated in all infertile men except those who were normozoospermic. However, FSH levels were found

TABLE NO. 5

Effect of *Mucuna pruriens* on reproductive hormonal profile of infertile men

Group	*Treatments*	*LH (mIU/mL)*	*Testosterone (ng/ml)*	*FSH (mIU/mL)*	*Prolactin (ng/ml)*
Control (n = 100)		7.85 ± 1.17	7.00 ± 0.67	5.86 ± 0.68	6.75 ± 0.71
Normozoospermic (n = 25)	Pre Treatment	6.39 ± 0.40[a]	5.86 ± 0.80[a]	6.67 ± 0.65[a]	6.93 ± 0.59[NS]
	Post Treatment[b]	7.88 ± 0.52	6.96 ± 0.62	5.71 ± 0.64	6.14 ± 0.67
Oligozoospermic (n = 25)	Pre Treatment[a]	4.10 ± 0.53	3.73 ± 0.40	8.15 ± 0.69	10.84± 1.22
	Post Treatment[b]	6.42 ± 0.63	5.29 ± 0.37	6.14 ± 0.73	7.39 ± 0.80
Asthenozoospermic (n = 25)	Pre Treatment[a]	3.96 ± 0.48	4.49 ± 0.87	6.82 ± 0.69	7.61 ± 0.75
	Post Treatment[b]	5.60 ± 0.53	5.72 ± 0.54	6.08 ± 0.88	6.82 ± 0.49
Azozoospermic (n = 25)	Pre Treatment	8.26±1.73[NS]	4.14±0.77[a]	13.43±2.77[a]	12.63±2.02[a]
	Post Treatment[b]	7.59±1.41	5.52±0.68	9.59±2.26	11.42±1.14

[a] $P < .01$ for all parameters compared with control groups (Dunnett test).

[b] $P < .01$ for all parameters compared with pretreatment groups (paired t test).erere

increased in infertile subjects who were normozoospermic (by 14%), oligozoospermic (by 39%), asthenozoospermic (by 16%) and azoospermic (by 129%). Similarly PRL also increased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 3, 61, 13 and 87%, respectively, as compared with that of fertile control men (Table 5).

Recovery of Hormonal Profile upon Treatment

Treatment with *M. pruriens* significantly increased the level of LH in all groups of infertile men except those who were azoospermic. LH levels were found increased by 23, 58 and 41% in normozoospermic, oligozoospermic and asthenozoospermic men, respectively, as compared with pre treatment values. Treatment also recovered the levels of T in normozoospermic (19%), oligozoospermic (42%), asthenozoospermic (27%) and azoospermic (33%) men significantly ($P<0.01$). Simultaneously, the treatment with *M. pruriens* also decreased the level of FSH and PRL in normozoospermic, oligozoospermic, asthenozoospermic and azoospermic groups by 14, 25, 11 and 29%, and 11, 32, 10 and 10%, respectively.

DNA Damage

The DNA damage parameters of the fertile group and the pre- and post-*M. pruriens* treated infertile subjects are shown from fig 20 to 24.

DNA damage associated with infertility

The DNA damage parameters i.e. Olive Tail Moment (OTM) (fig. 20), mean percentage of tail DNA (fig. 21) and tail length (fig. 22) were found significantly increased in all groups of infertile individuals whereas the percentage of head DNA content was found decreased as compared with control (fig. 23). A marked increase in

OTM (306%), mean percentage of tail DNA (188%) and tail length (70%) and significant decrease in head DNA content (-23%) was observed in oligozoospermic infertile men.

Protection against DNA damage reduced upon treatment

After treatment with *M. pruriens* the percentage of head DNA content was found increased significantly in normozoospermic, oligozoospermic, and asthenozoospermic men as compared with the pretreatment parameters and these effects were found more pronounced in oligozoospermic (21.75%) and asthenozoospermic men (21.67%) Following treatment the percentage of tail DNA and tail length were significantly decreased ($P<.01$) in all groups of infertile men. Moreover, the OTM was also significantly decreased ($P<.01$) in all infertile groups. Most significant decrease in OTM (-54%) and tail DNA content (48%) was observed in oligozoospermic men and tail length (35%) in asthenozoospermic infertile men.

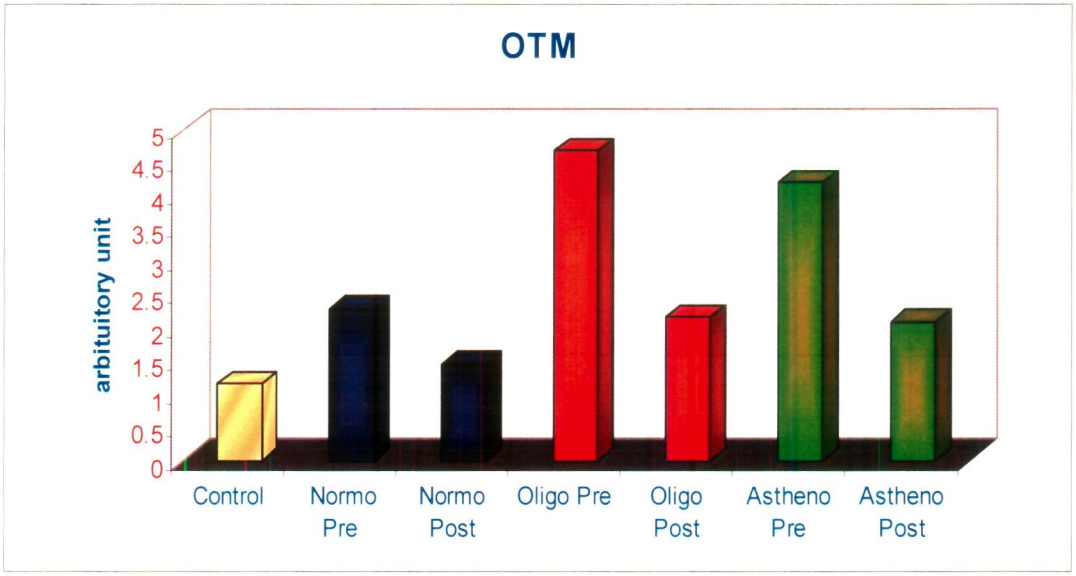


Figure 20: Effect of *M. pruriens* on spermatozoa Olive Tail Moment of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

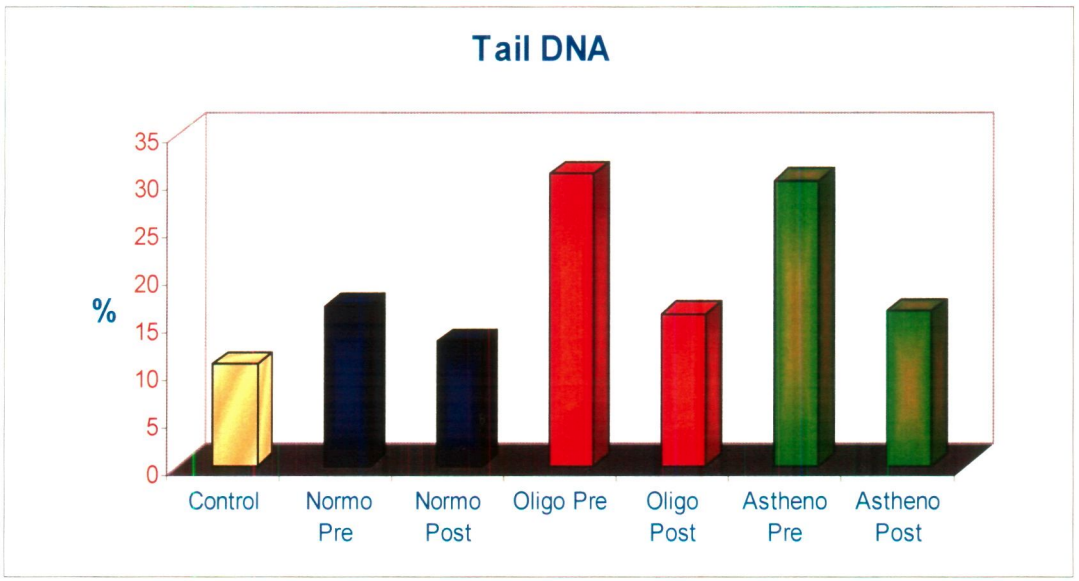


Figure 21: Effect of *M. pruriens* on spermatozoa Tail DNA of infertile men
Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

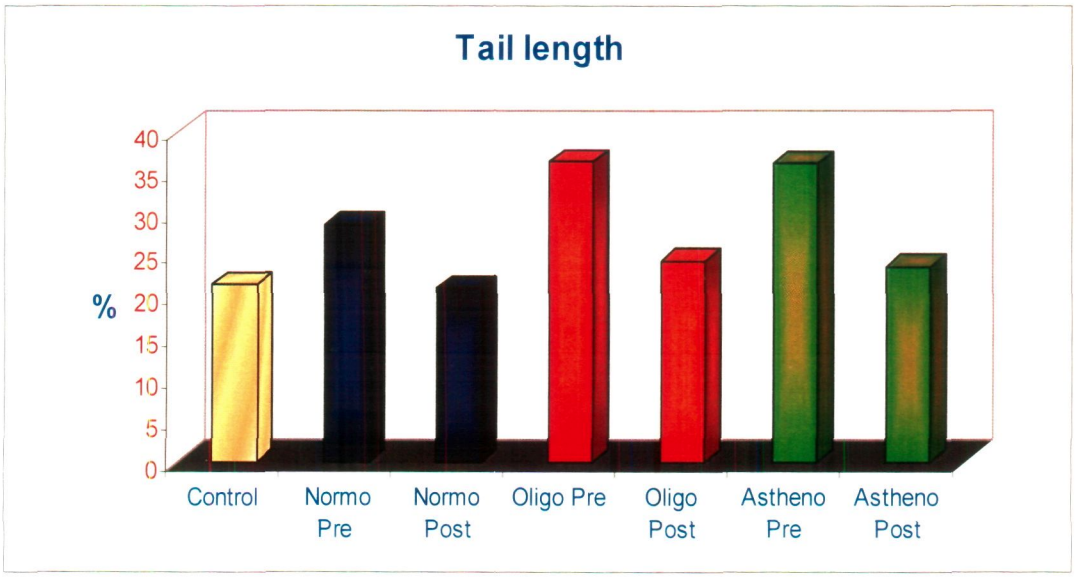


Figure 22: Effect of *M. pruriens* on spermatozoa DNA Tail length of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

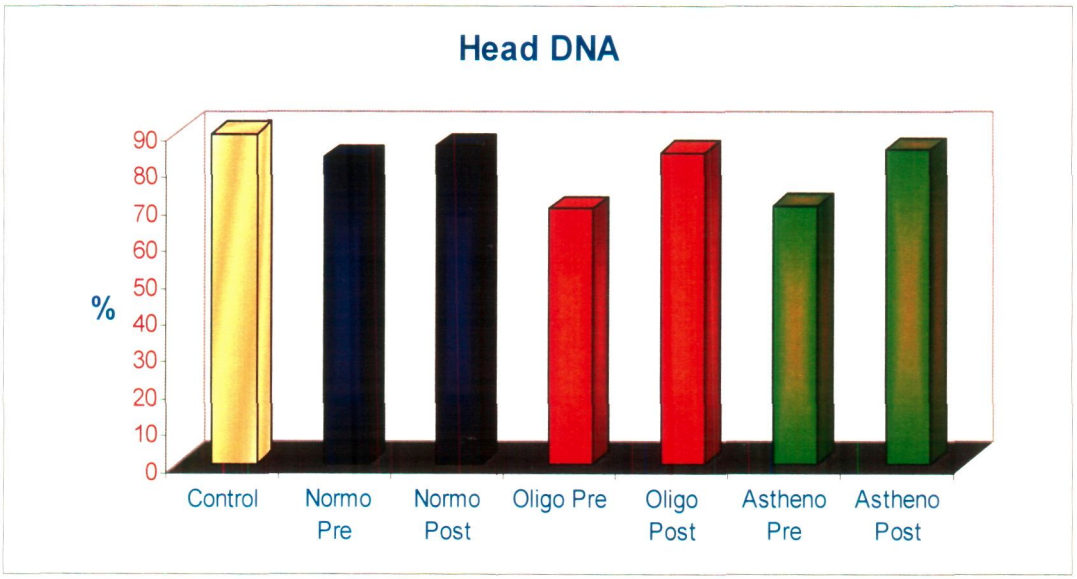


Figure 23: Effect of *M. pruriens* on spermatozoa Head DNA of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

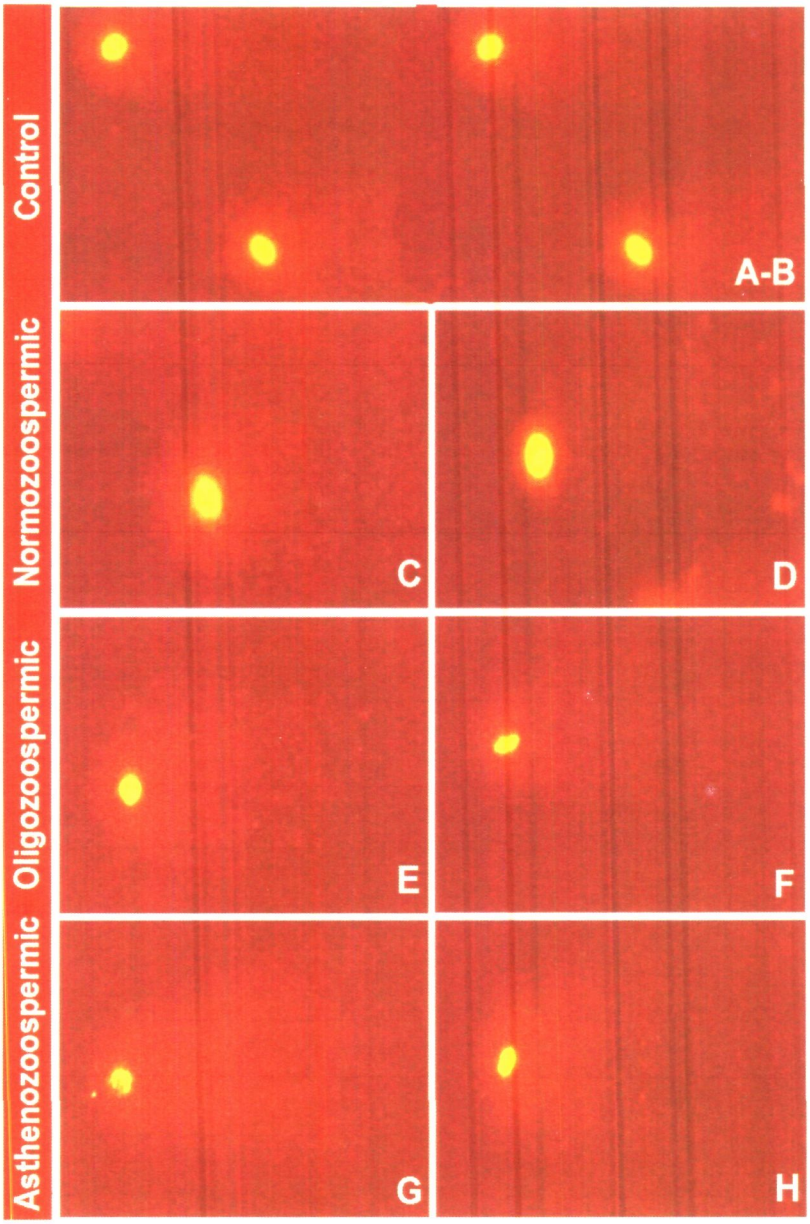


Figure 24: COMET assay

Effect of *M. pruriens* on spermatozoal DNA damage of infertile men.

Panel A-B shows spermatozoa from control (normozoospermic fertile) man. Panel C and D show spermatozoa from normozoospermic infertile man, pre-treatment and post-treatment, respectively; panel E and F show spermatozoa from oligozoospermic individual, pre-treatment and post-treatment, respectively; panel G and H show spermatozoa from asthenozoospermic individual, pre-treatment and post-treatment, respectively.

WITHANIA SOMNIFERA

Semen Profile:

The semen profiles of the fertile (control) group and the pre- and post-*W. somnifera* treated infertile groups are shown in Table 6.

Semen Profile Associated With Male Infertility

In normal healthy fertile men (control groups), sperm concentration ($x10^6$) was 79.00 ± 15.37 /ml, sperm count ($x10^6$) per ejaculate was 223.00 ± 52.25, motility was 72.67 ± 7.63 % and semen volume was 2.84± 0.45 ml. However, in infertile normozoospermic, oligozoospermic and asthenozoospermic men, there was a decline in their sperm concentration by 32, 88 and 46% and sperm per ejaculate by 46, 92 and 54%, motility by 25, 28 and 78%, following decrease in semen volume by 22, 34 and 30%, respectively. The semen volume was found decreased in azoospermic men by 13%, as compared with control.

Treatment with W. somnifera Improve Semen Profile

Treatment of the abovementioned infertile men with *Withania somnifera root* powder (5gm/day) for three months showed significant reversal of above semen parameters. Our results showed that following treatment the sperm concentration of normozoospermic, oligozoospermic and asthenozoospermic infertile men were increased by 33, 18, and 32%, respectively. Similarly, sperms per ejaculate were also found increased by 60, 264 and 37%, as compared with their pre treatment parameters. Motility of spermatozoa of normozoospermic, oligozoospermic and asthenozoospermic infertile men was found increased by 17, 18 and 48%, respectively as compared to pre treatments values and semen volume by 20, 30, 4 and 7 % in normozoospermic,

TABLE NO. 6

Effect of *Withania somnifera* on semen profile of infertile men

Group	*Treatments*	*Semen volume (mL)*	*Sperm concentration (1×10^6 / mL)*	*Sperm count ($1x10^6$)per ejaculate*	*Motility (%)*
Control (n = 75)		2.84 ± 0.45	79.00 ± 15.37	223.00 ± 52.25	72.67 ± 7.63
Normozoospermic (n = 25)	Pre Treatment[a]	2.21 ± 0.43	54.04 ± 8.80	119.37 ± 29.65	54.17 ± 10.18
	Post Treatment[b]	2.65 ± 0.39	71.88 ± 10.70	191.38 ± 42.12	63.54 ± 10.05
Oligozoospermic (n = 25)	Pre Treatment[a]	1.86 ± 0.37	9.78 ± 1.89	18.06 ± 4.74	52.5 ± 8.47
	Post Treatment[b]	2.42 ± 0.30	27.32 ± 5.67	65.63 ± 13.52	62.71 ± 9.32
Asthenozoospermic (n = 25)	Pre Treatment[a]	2.44 ± 0.45	43.49 ± 8.84	103.37 ± 17.65	16.44 ± 2.91
	Post Treatment	2.54 ± 0.54 [NS]	57.6 ± 11.79[b]	141.91 ± 21.95[b]	24.44 ± 4.48[b]
Azozoospermic (n = 25)	Pretreatmen	2.48±0.61 a*	Nil	Nil	NA
	Posttreatment	2.67±0.58[NS]	Nil	Nil	NA

[a]$P < .01$ for all parameters compared with control (Dunnett test).
[b]$P < .01$ for all parameters compared with pretreatment (paired t test).
NS = Not significant, NA = Not applicable.

oligozoospermic, asthenozoospermic and azoospermic, respectively, as compared with their pre treatment values. Whereas treatment didn't recovered the level of sperm concentration in azoospermic infertile men.

Status of Lipid Profile in Seminal Plasma of Infertile Men

The lipid profiles of infertile subjects and controls are listed in Table 7.

The levels of total lipids, cholesterol, triglycerides, and phospholipids in the seminal plasma of fertile men were 362.57 ± 38.37 mg/dL, 57.25 ± 6.24 mg/dL, 59.83 ± 4.83 mg/dL, and 169.17 ± 12.73 mg/dL, respectively. These levels were significantly reduced in all groups of infertile men when compared with controls. The level of total lipids decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 24, 27, 37 and 34%, and cholesterol by 20, 11, 13 and 25%, respectively. Similarly triglycerides levels were found decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 12, 29, 33 and 27%, and phospholipids level by 17, 28 24 and 25%, respectively.

Treatment Restored the Levels of Lipid Profile in Seminal Plasma

Treatment with *W. somnifera* recovered the level of total lipids in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 18, 16, 25 and 14%, and cholesterol levels by 16, 11, 10 and 10%, respectively, as compared with pre treatment values. Similarly, treatment also recovered the levels of triglycerides in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 9, 16, 20 and 12%, and phospholipids by 13, 21, 18 and 12%, respectively.

TABLE NO. 7

Effect of *Withania somnifera* on seminal lipid profile of infertile men

Group	*Treatments*	*Total lipids (mg/dL)*	*Cholesterol (mg/dL)*	*Triglycerides (mg/dL)*	*Phospholipids (mg/dL)*
Control (n =100)		362.57 ± 38.37	57.25 ± 6.24	59.83 ± 4.83	169.17 ± 12.73
Normozoospermic (n = 25)	Pre Treatment[a]	274.43 ± 22.34	45.28 ± 3.97	52.72 ± 3.83	139.82 ± 11.83
	Post Treatment[b]	323.57 ± 28.64	52.49 ± 3.72	57.62 ± 4.72	157.82 ± 14.83
Oligozoospermic (n = 25)	Pre Treatment[a]	264.36 ± 25.82	52.73 ± 6.75	42.68 ± 3.82	121.29 ± 14.62
	Post Treatment[b]	307 ± 31.45	56.37 ± 9.26	49.38 ± 3.83	147.85 ± 13.83
Asthenozoospermic (n = 25)	Pre Treatment[a]	228.47 ± 19.53	49.85 ± 4.69	39.93 ± 2.83	129 ± 13.45
	Post Treatment[b]	284.37 ± 25.62	54.53 ± 8.45	47.62 ± 3.87	152.83 ± 13.88
Azozoospermic (n = 25)	Pre Treatment[a]	239.83 ± 23.41	42.64 ± 5.32	43.62 ± 3.51	126.73 ± 12.83
	Post Treatment[b]	273.63 ± 21.52	46.73 ± 3.74	48.85 ± 5.63	141.83 ± 15.86

[a] $P < 0.01$ for all parameters compared with control groups (Dunnett test).

[b] $P < 0.01$ for all parameters compared with pretreatment groups (paired t test).

Oxidative Biomarkers and Male Infertility

It was observed that the level of lipid peroxides (LPO) in seminal plasma of control healthy fertile men was 1.74±0.19 nmol MDA/ml, however, these levels in infertile normozoospermic, oligozoospermic and asthenozoospermic men, were increased by 87, 44 and 63%, respectively, while it was found decreased in azoospermic men by 27% (fig. 25). The level of protein carbonyls in the seminal plasma of fertile men was 1.96±0.54 nmol/mg protein. This parameter was found increased in the seminal plasma of normozoospermic, oligozoospermic and asthenozoospermic infertile men by 59, 94 and 43 % respectively, and decreased in azoospermics by 24% (fig. 26). The infertile men had significantly high mean percentage of ROS in the ejaculated spermatozoa samples than the control samples and increament was found in normozoospermic, oligozoospermic and asthenozoospermic, infertile men by 130, 205 and 172%, respectively (fig. 27). The level of total antioxidant capacity (TAC) in the seminal plasma of fertile men was 1.63±0.09 nM trolox equivalent. TAC was found decrease in infertile groups of normozoospermic men by 21%, in oligozoospermic men by 42%, asthenozoospermic men by 47% and azoospermic men by 40% (fig. 28).

Treatment with W. somnifera Reduced Oxidative Biomarkers

After treatment with *W. somnifera* for three months the levels of LPO in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic infertile men, were decreased by 22, 17, 29 and 12%, respectively, as compared with pre treatment parameters (fig. 25). Similarly the levels of protein carbonyls in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic

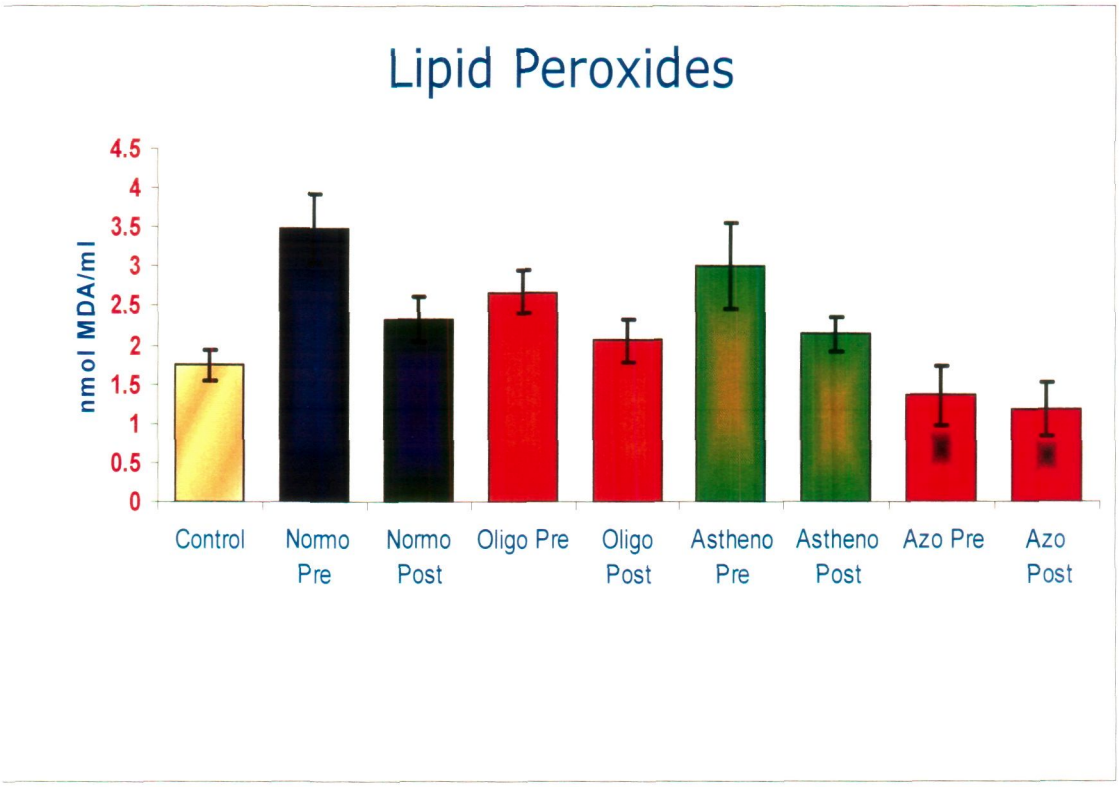


Figure 25: Effect of *W. somnifera* on Lipid peroxide levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

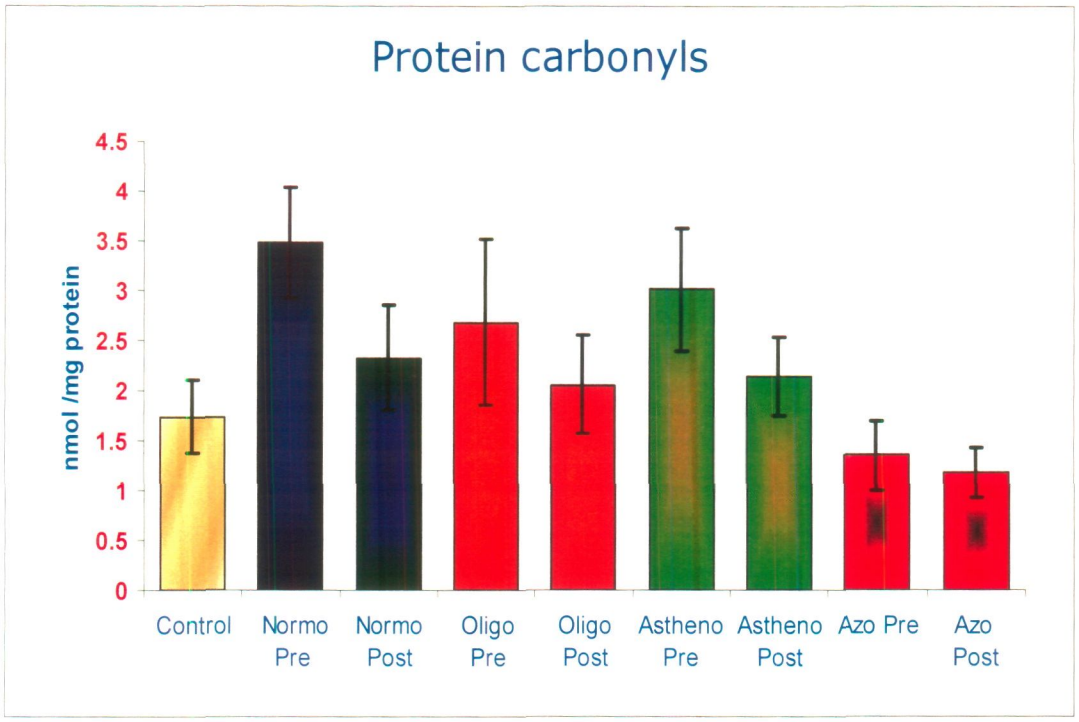


Figure 26: Effect of *W. somnifera* on Protein carbonyl levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

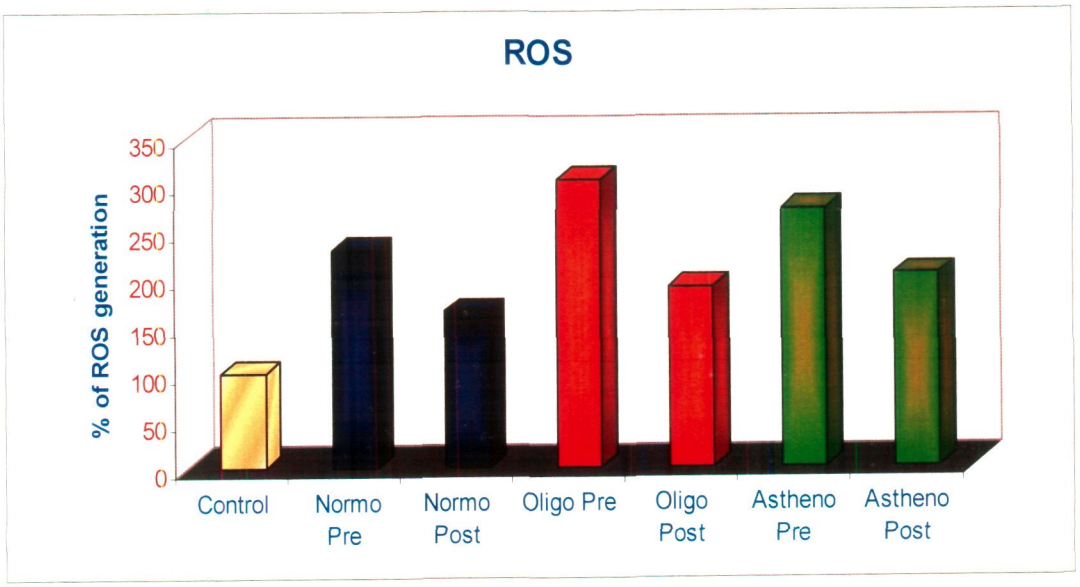


Figure 27: Effect of *W. somnifera* on levels of ROS in spermatozoa of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

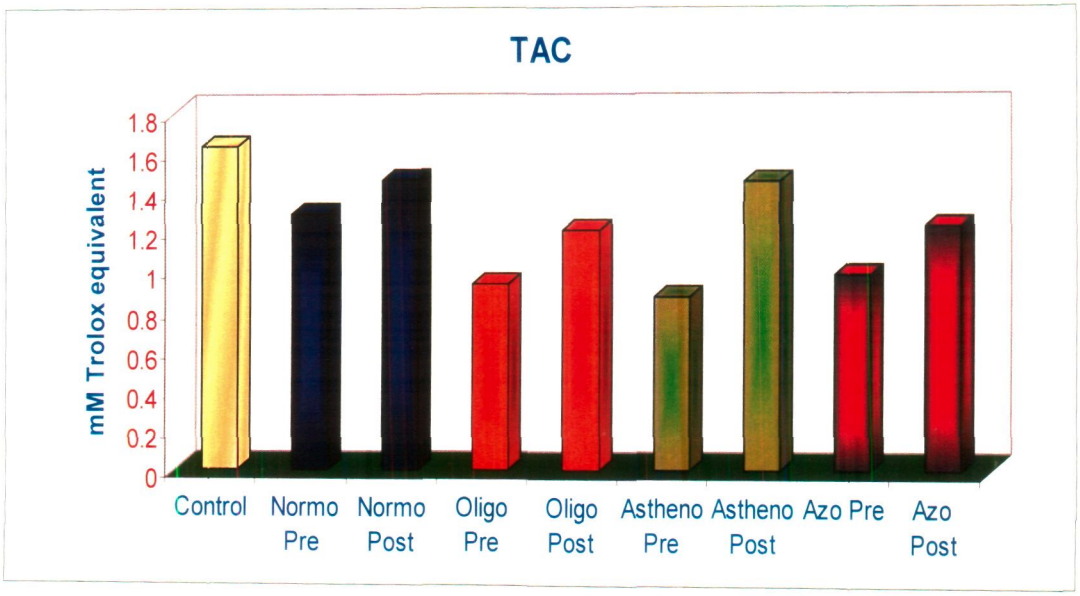


Figure 28: Effect of *W. somnifera* on the levels of seminal TAC of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

and azoospermic infertile men were decreased by 29, 20, 20 and 24%,, respectively (fig. 26). The mean percentage of ROS in the ejaculated spermatozoa was also significantly decreased upon treatment as compared with their pre treatment values and the decrease was found in normozoospermic, oligozoospermic and asthenozoospermic, infertile men by 28, 38 and 25%, respectively (fig. 27). Treatment with *W. somnifera* increases the levels of TAC in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 13, 29, 66 and 26%, respectively (fig. 28).

Status of Antioxidant Enzymes Male Infertile

The antioxidant enzymes of the fertile (control) group and the pre- and post- *W. somnifera* treated infertile groups are depicted in Table 8.

We observed that superoxide dismutase (SOD) activity in seminal plasma of healthy fertile controls was 8.17±0.71 unit/mg protein. However, enzyme activity was found suppressed in infertile normozoospermic men by 18%, in oligozoospermic men by 32%, asthenozoospermic men by 36% and azoospermic men by 37%. Similarly, the activity of catalase in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was also found suppressed by 14, 13, 31 and 42%, respectively, as compared with that of fertile control group (9.18±0.93 unit/mg protein).

The glutathione reductase (GR) and glutathione peroxidase (GPx) activity in seminal plasma of fertile men was 15.13± 3.49 U/min/mg protein and 8.62±2.84 U/min/mg protein respectively. However, these enzyme activities were found suppressed in infertile

Table 8: *Effect of Withania somnifera on enzyme activity of infertile men.*

Group	*Treatments*	*SOD U/mg protein*	*Catalase U/mg protein*	*GR U/min/mg protein*	*GPx U/min/mg protein*
Control (n = 100)		8.17±0.71	9.18±0.93	15.13±3.49	8.62±2.84
Normozoospermic (n = 25)	Pretreatment	6.71±0.88 a*	8.20±1.26 a*	13.50±2.68 NS	7.28±1.43 NS
	Posttreatment	7.36±0.77 b*	10.22±1.80 b*	16.34±2.50 b*	9.18±2.13 b*
Oligozoospermic (n = 25)	Pretreatment	5.54±0.85 a**	8.31±1.16 a*	13.07±2.80 a*	7.13±8.91 a*
	Posttreatment	6.95±1.11 b*	9.98±1.00 b*	15.22±3.21 b*	8.91±1.91 b*
Asthenozoospermic (n = 25)	Pretreatment	5.27±0.70 a**	6.58±0.96 a**	12.31±3.14 a*	6.85±1.50 a*
	Posttreatment	6.50±0.95 b*	8.22±2.01 b**	14.08±2.45 b*	8.23±1.39 b*
Azozoospermic (n = 25)	Pretreatment	5.12±0.59 a**	5.49±0.72 a**	13.08±2.59 a*	6.56±1.33 a*
	Posttreatment	6.02±0.88 b*	7.25±0.73 b**	14.50±2.87 b*	7.90±1.68 b*

a* P<.05, a** P<.01 as compared with control (Dunnett test).
b* P<.05, b** P<.01 as compared with pretreatment (paired t test). NS= Non significant

normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 13, 16, 21 and 16% and 13, 15, 18 and 22 %, respectively (Table 8).

Antioxidant Enzymes Activity Recovered Upon Treatment With W. somnifera

Treatment with *W. somnifera* rerecovered SOD activity in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 9, 25, 23 and 17%, and catalase activity by 24, 20, 25 and 32%, respectively, as compared with pre treatment values. Similarly, treatment also recovered GR activity in seminal plasma of normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 21, 16, 14 and 10%, and GPx activity by 26, 24, 20 and 20%, respectively. The catalase and GPx activities were not found recovered significantly in azoospermic men.

Male Infertility and the Levels of Antioxidant Vitamins in Seminal Plasma

The levels of vitamin A in seminal plasma of control fertile group were 28.61± 4.43 µg/dl, which were found decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 35, 39, 45 and 51%, respectively (fig. 29). Furthermore, the level of vitamin E in seminal plasma of infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was found decreased by 22, 36, 44 and 47%, respectively, as compared with that of control fertile men (0.143±0.012 mg/dl) (fig. 30). The level of vitamin C in seminal plasma of control groups was 5.85± 0.73 mg/dl which was found decreased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 28, 15, 13 and 23%, respectively (fig. 31).

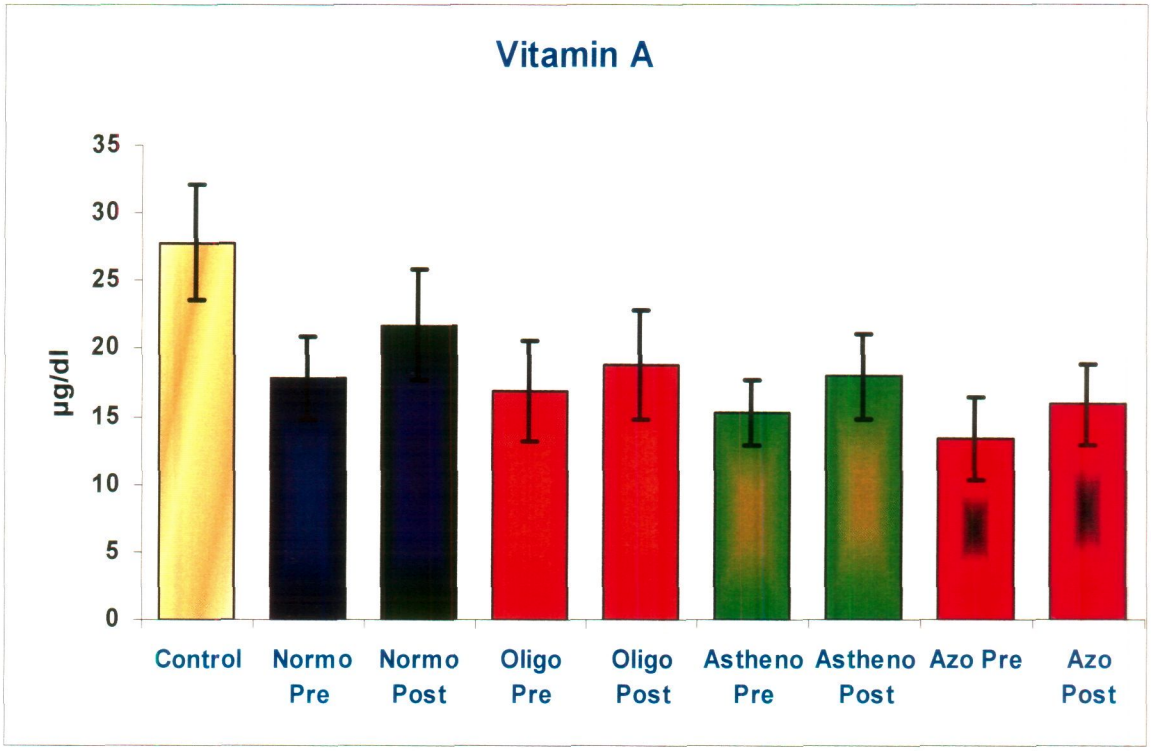


Figure 29: Effect of *W. somnifera* on Vitamin A levels in seminal plasma of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

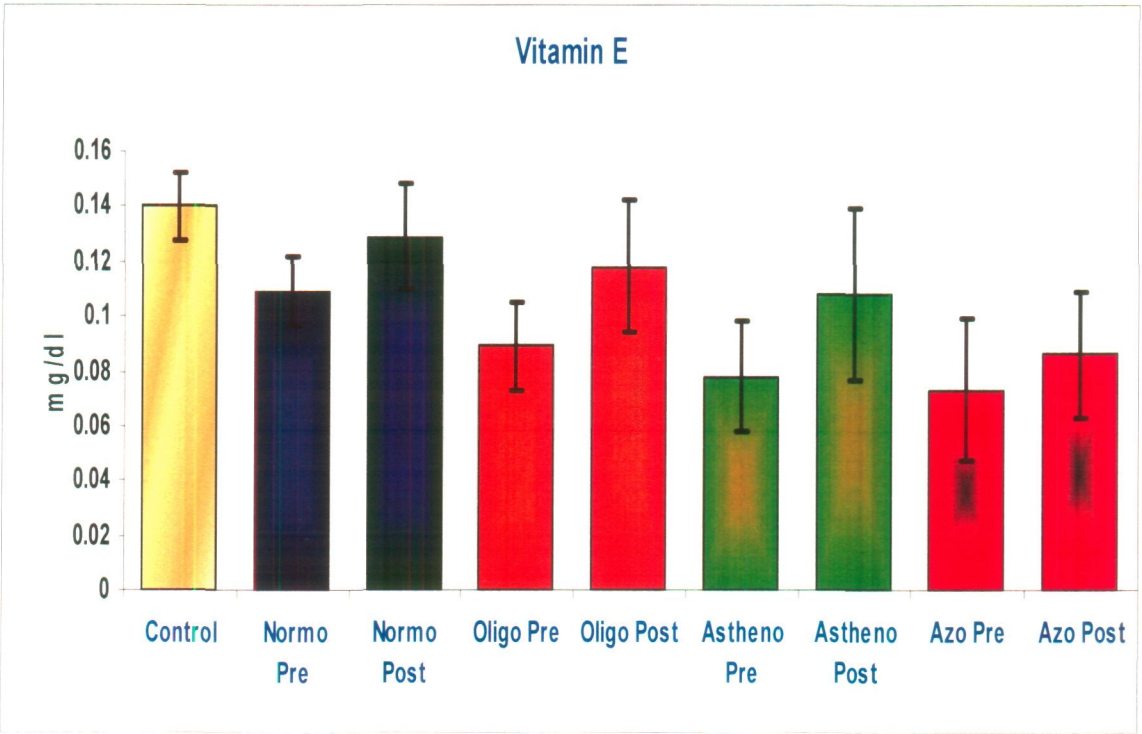


Figure 30: Effect of *W. somnifera* on Vitamin E levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

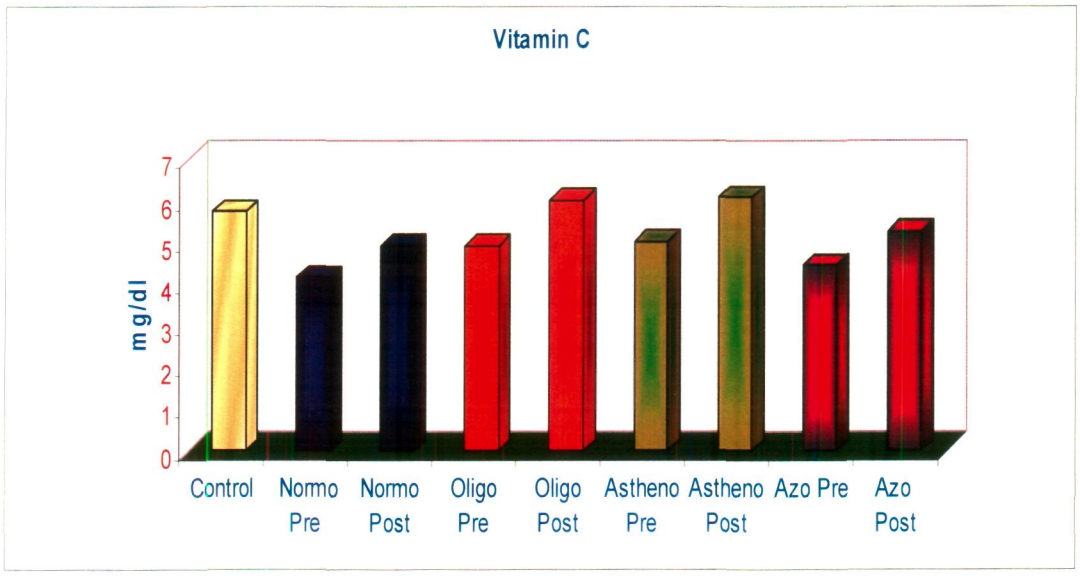


Figure 31: Effect of *W. somnifera* on Vitamin C levels in seminal plasma of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

Treatment Recovered the Levels of Antioxidant Enzymes

Treatment with *W. somnifera* in normozoospermic, oligozoospermic, asthenozoospermic and azozoosermic men showed recovery in the seminal plasma levels of vitamin A by 21, 11, 17 and 18% respectively (fig. 29). The level of vitamin E in seminal plasma was found recovered by 18, 32, 38 and 17%, respectively as compared with pre-treated values (fig. 30). Similarly, the treatment also recovered the level of vitamin C in seminal plasma by 18, 21, 20 and 18% respectively, as compared with pre treatment values (fig. 31).

Hormonal Profile Associated With Male Infertility

Mean basal hormone levels of infertile subjects and controls are listed in Table 9.

Luteinizing hormone and Testosterone

The serum LH concentration in control group was 7.94 ± 1.00 mIU/mL. This parameter was significantly lower in all infertile men except those who were azoospermic. LH levels were found decreased in infertile normozoospermic, oligozoospermic and asthenozoospermic men by 13, 50 and 52%, respectively, and increased in azoospermic men by 7%. Furthermore, the level of Serum T of infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men was found decreased by 18, 51, 39 and 48%, respectively, as compared with that of control fertile men (7.09±0.63 ng/ml).

Follicle-stimulating hormone and Prolactin

FSH and PRL levels in the serum of fertile control men were 5.67 ± 0.91 mIU/mL and 7.10 ± 0.67 ng/mL, respectively. These parameters were significantly elevated in all

infertile men except those were normozoospermic. However, FSH levels were found increased in infertile subjects who were normozoospermic (by 7%), oligozoospermic (by 37%), asthenozoospermic (by 14%) and azoospermic (by 128%). Similarly, PRL also increased in infertile normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 2, 49, 10 and 85%, respectively, as compared with that of fertile control parameters (Table 9).

Reversal in the Level of Hormonal Profile upon Treatment

Treatment significantly increased the level of LH in all groups of infertile men except those who were azoospermic. LH levels were found increased by 14, 49 and 41% in normozoospermic, oligozoospermic and asthenozoospermic men, respectively, as compared with pre treatment values. Treatment also recovered the levels of T in normozoospermic (18%), oligozoospermic (51%), asthenozoospermic (39%) and azoospermic (27%) men significantly ($P<0.01$). After treatment with *W. somnifera,* FSH and PRL levels were reduced significantly in all groups of infertile men except those who were normozoospermic. The FSH levels were found decreased in normozoospermic, oligozoospermic, asthenozoospermic and azoospermic men by 10, 19, 8 and 15%, and PRL by 3, 17, 8 and 11%.

TABLE NO.9

Effect of *Withania somnifera* on hormonal profile in serum of infertile males

Group	*Treatments*	*LH (mIU/mL)*	*Testosterone (ng/mL)*	*FSH (mIU/mL)*	*Prolactin (ng/mL)*
Control (n = 100)		7.94 ± 1.00	7.09 ± 0.63	5.67 ± 0.91	7.10 ± 0.67
Normozoospermic (n = 25)	Pre Treatment	6.87 ± 0.60**	5.80 ± 0.88**	6.07 ± 0.69^{NS}	7.21 ± 0.72^{NS}
	Post Treatment	7.85 ± 0.53^{b}	6.65 ± 0.78^{b}	5.75 ± 0.60^{a}	6.93 ± 0.67^{NS}
Oligozoospermic (n = 25)	Pre Treatment	4.02 ± 0.57**	3.51 ± 0.56**	7.78 ± 0.77**	10.57 ± 1.42**
	Post Treatment	5.98 ± 0.80^{b}	4.94 ± 0.54^{b}	6.27 ± 0.76^{b}	8.75 ± 1.28^{b}
Asthenozoospermic (n = 25)	Pre Treatment	3.82 ± 0.59**	4.32 ± 0.89**	6.49 ± 0.85**	7.78 ± 0.82*
	Post Treatment	5.37 ± 0.61^{b}	5.23 ± 0.80^{b}	5.95 ± 0.96^{a}	7.19 ± 0.82^{b}
Azozoospermic (n = 25)	Pre Treatment	8.49 ± 2.13^{NS}	3.92±0.82**	12.94±3.10**	13.12±2.67**
	Post Treatment	7.82 ± 1.62^{a}	4.97 ± 0.93^{a}	10.53 ± 2.83^{a}	11.69 ± 2.13^{a}

(Dunnett test).

(paired t test). NS = Not significant..

DNA Damage

The DNA damage parameters of the fertile group and the pre- and post-*W. somnifera* treated infertile subjects are shown from fig. 32 to 36.

DNA damage associated with infertility

The DNA damage parameters i.e. Olive Tail Moment (OTM) (fig. 32), mean percentage of tail DNA (OTM) (fig. 33) and tail length (OTM) (fig. 34) were found significantly increased in all groups of infertile men whereas the percentage of head DNA content was found decreased as compared with control (OTM) (fig. 35). Most significant increase in OTM (286%) and mean percentage of tail DNA (176%) was observed in oligozoospermic men, whereas tail length (48%) in normozoospermic and significant decrease in head DNA (-25%) was observed in oligozoospermic infertile men.

Protective effect of W. somnifera against DNA damage.

Upon treatment the percentage of head DNA content was found increased significantly in normozoospermic, oligozoospermic, and asthenozoospermic men as compared with the pretreatment parameters and most significant increase was observed in oligozoospermic men (18%). Percentage of tail DNA, tail length and OTM were significantly decreased ($P<0.01$) in all groups of infertile individuals after treatment Most significant decrease in OTM (37%), tail DNA (-39%) and length (-27 %) was observed in oligozoospermic infertile men.

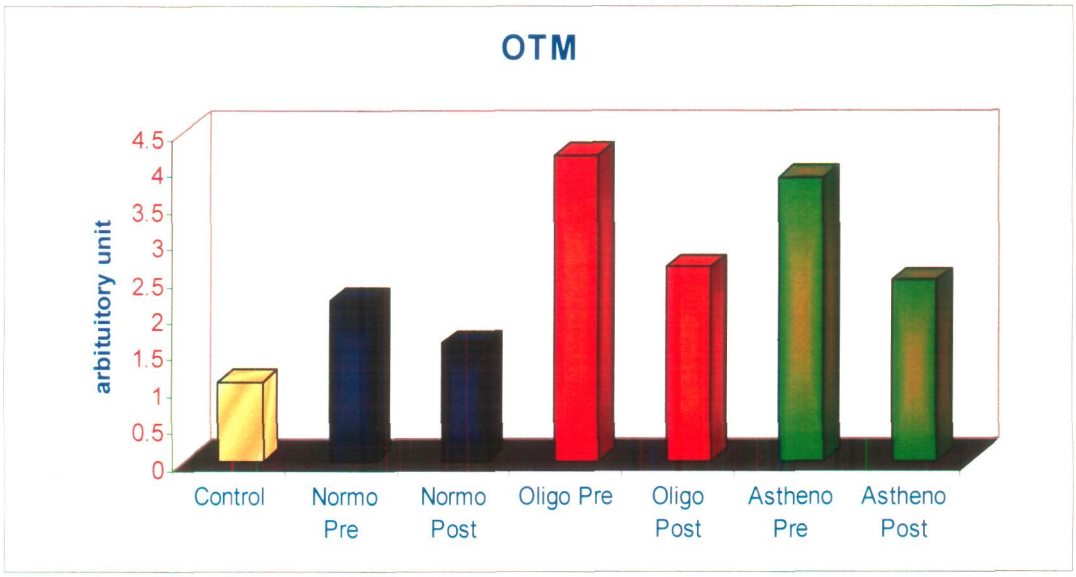


Figure 32: Effect of *W. somnifera* on spermatozoa Olive Tail Movement of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

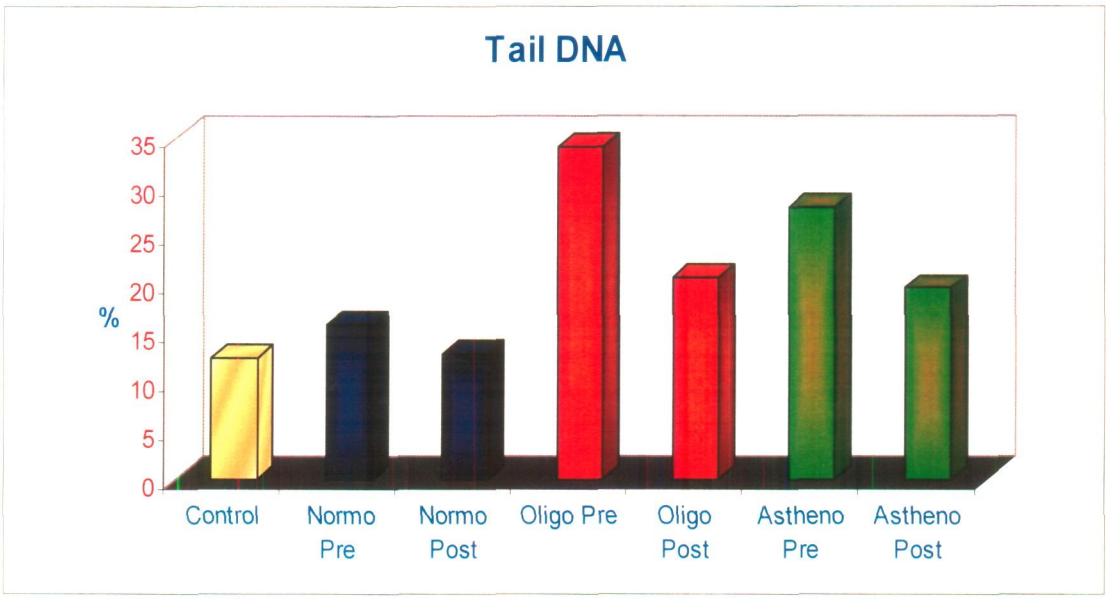


Figure 33: Effect of *W. somnifera* on spermatozoa Tail DNA of infertile men.

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

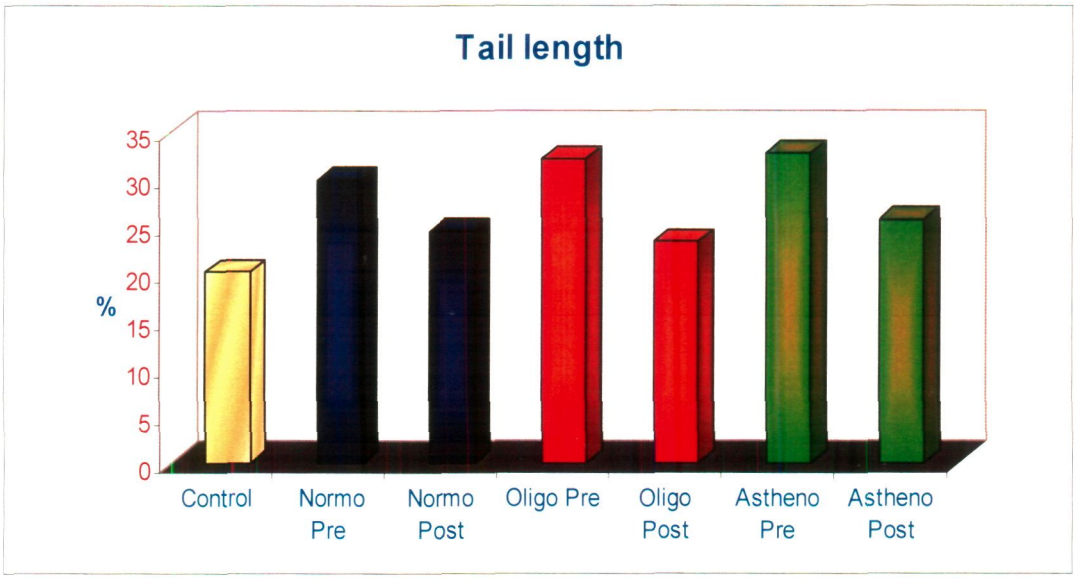


Figure 34: Effect of *W. somnifera* on spermatozoa DNA Tail length of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

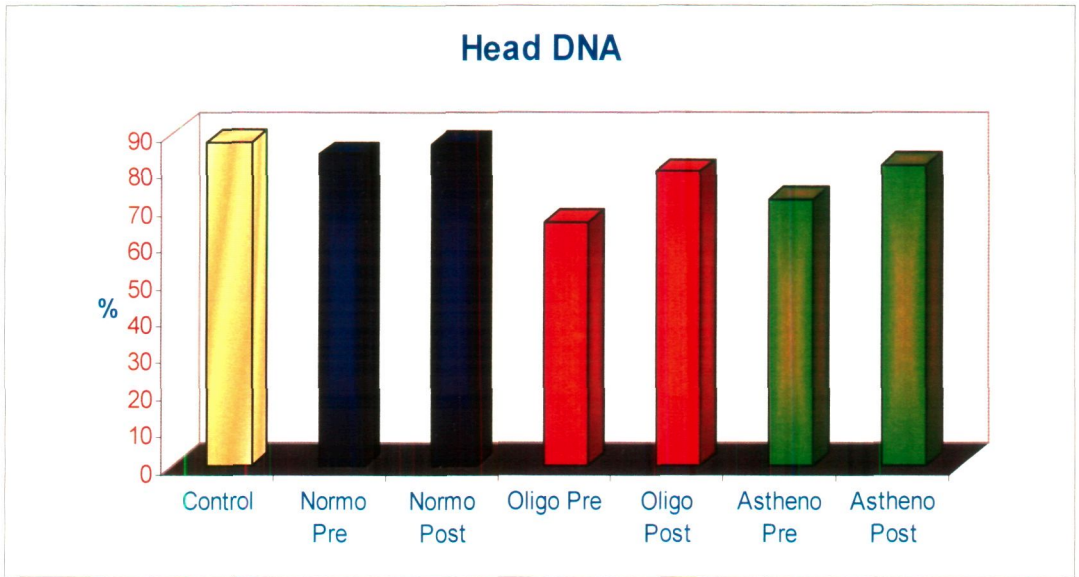


Figure 35: Effect of *W. somnifera* on spermatozoa Head DNA of infertile men

Normo: Normozoospermic, Oligo: Oligozoospermic, Astheno: Asthenozoospermic, Azo: Azoospermic, Pre: Pre-treatment, Post: Post-treatment.

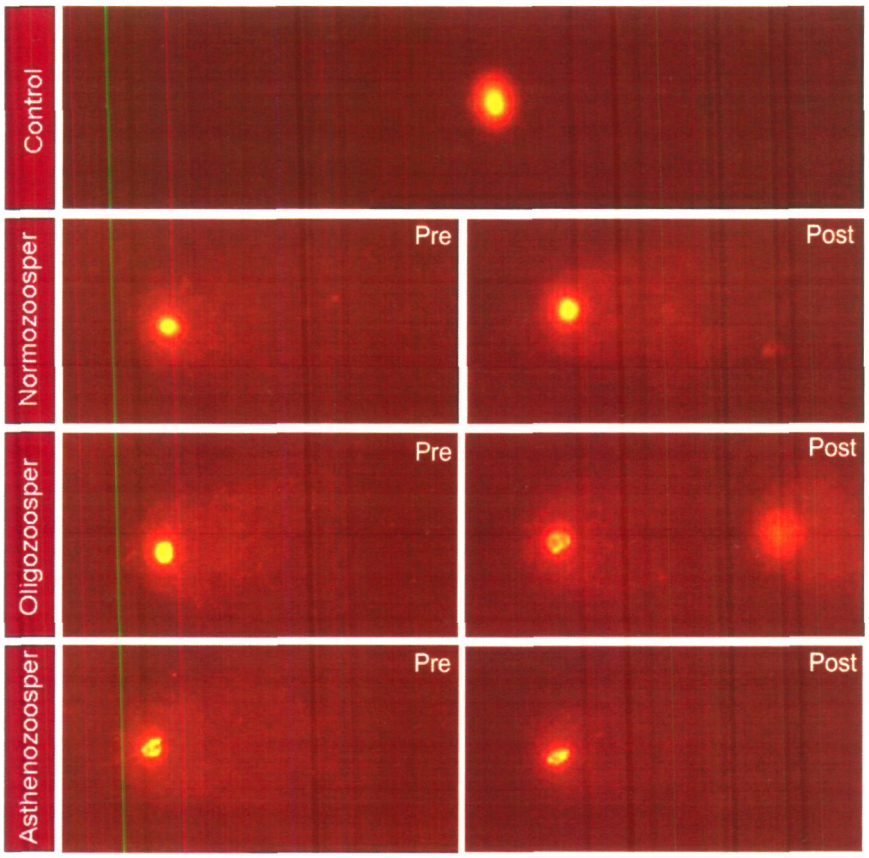


Figure 36: COMET assay.

Effect of *W. somnifera* on spermatozoal DNA damage of infertile men.

Pre: Pre-treatment, Post: Post-treatment.

Discussion

The results of our study demonstrated that male infertility is associated with abnormal semen profile i.e. low sperm count, motility and semen volume along with disturbance in the levels of seminal lipid profiles. We also observed that spermatozoa of infertile men produce significantly high levels of ROS and show more DNA damage as compared with fertile men. The same is reflected by elevated levels of seminal lipid peroxides, protein carbonyl groups and decreased seminal total antioxidant capacity (TAC). The condition was further complexed by reduced activity of antioxidant enzymes namely SOD, catalase, glutathione reductase and glutathione peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we also observed hormonal imbalance i.e., low level of testosterone and LH, and elevated levels of FSH and PRL, in all groups of infertile men.

Treatment of infertile subjects with *M. pruriens* for three months significantly improved the sperm concentration and motility. Earlier, it has been reported that *M. pruriens* helps by some central mechanism to increase secretion of semen, decrease spermatorrhoea and to act as restorative agent, invigorator tonic and aphrodisiac in diseases characterized by weakness or loss of sexual power (Nandkarni, 1986). The biological basis and exact mechanism of action of *M. pruriens* on infertility is not well known. However, the beneficial effect may be attributed to its antioxidant and neurostimulatory properties (Misra and Wagner, 2007). *M. pruriens* is reported to contain many bioactive constituents, including alkaloids, coumarins, flavonoids and alkylamines etc. which may play an important role in increasing the antioxidant capacity of treated men. There are also reports that the methanol extract of *M. pruriens* seeds have strong antioxidant activity, as it inhibits 1,1-diphenyl-2-picryl-hydrazyl (DPPH), hydroxyl radical and it also has nitric oxide and superoxide anion

scavenging, hydrogen peroxide decomposing and reducing power (Rajeshwar *et al.*, 2005).

The other herb *W. somnifera* included in the objective of this study was also evaluated in infertile men in a similar manner. We observed that treatment of infertile men with *W. somnifera* for three months significantly improved their sperm concentration and motility. *W. somnifera* also improved the activity of antioxidant enzymes and recovered the levels of TAC. Moreover, it reduced the seminal lipid peroxides and protein carbonyl groups along with ROS levels in spermatozoa of infertile men. Complementing our findings, earlier studies have reported that *W. somnifera* inhibits lipid peroxidation in stress induced animals (Dhuley, 1998).

The detrimental effects of reactive oxygen species (ROS) on spermatozoa were suggested more than 65 years ago with the demonstration that exposure of sperm to oxygen results in sperm toxicity (McLeod, 1943). It has been reported that semen of infertile males contains significantly high levels of ROS, as has been observed by us also, whereas fertile men do not have detectable levels of semen ROS (Venkatesh *et al.*, 2009). There are several reports that the ROS produced by leukocytes and/or by spermatozoa have deleterious effects on sperm function (Aitkin, 1994). The half-life of ROS is very short and it is difficult to detect ROS in semen directly. However, malondialdehyde (MDA), one of the lipid peroxidative end-products produced by ROS when it attacks sperm membrane, can indirectly reflect the damage of sperm (Ben Abdallah *et al.*, 2009). Therefore, the determination of MDA concentration in seminal plasma may be taken as one of important markers for the diagnosis and treatment of male infertility induced by excessive lipid peroxidation (Shang *et al.*, 2004; Li *et al.*, 2004). We observed elevated ROS and lipid peroxide levels in all infertile men and also found that both the herbs used in the study (*M. pruriens* and

W. somnifera) significantly decreased the lipid peroxides and MDA levels in infertile males.

The susceptibility of human spermatozoa to oxidative stress stems from the abundance of unsaturated fatty acids in the sperm plasma membrane. These unsaturated fatty acids provide fluidity that is necessary for sperm motility and membrane fusion events such as the acrosome reaction and sperm-egg interaction, both required for natural fertilization. However, the unsaturated nature of molecules predisposes them to free radical attack and ongoing lipid peroxidation throughout the sperm plasma membrane. Once this process has been initiated, accumulation of lipid peroxides occurs on the sperm surface which ensures sperm dysfunction and sperm death (Sikka, 2004; Alvarez *et al.,* 1987), thus lipid peroxidation has a deleterious effect on the semen quality (Tavilani *et al.,* 2005). Along with the lipid peroxides and ROS, several studies have demonstrated that infertile men are more likely than fertile ones to have depressed TAC and lower levels of individual antioxidants (Mahfouz *et al.,* 2009; Pasqualotto *et al.,* 2008). Moreover, our results showed that treatment with *M. pruriens* and *W. somnifera* significantly decreased the levels of ROS in spermatozoa and seminal protein carbonyl groups along with increasing the levels of seminal TAC.

Lipids constitute major components of cellular membranes and they play an important role in maintaining the structural and functional integrity of the spermatozoa. The seminal plasma is characterized by abundance of cholesterol and phospholipids, mainly sphingomyelins and phosphatidyl ethanolamines, present for the most part in the form of HDL lipids (Vignon *et al.,* 1989). Their secretion is hormone dependent (Vignon *et al.,* 1991). The part played by lipids in capacitation is

better understood and the particular characteristic of lipid composition in seminal fluid allows a successful penetration of ovum by spermatozoa (Gadella *et al.,* 2008).

Our results showed a significant reduction in total lipid levels in all infertile groups as compared with controls. These results are similar to those reported earlier in chronic alcoholic infertile men (Gomathi *et al.,* 1993), patients with chronic infections (Vignon *et al.,* 1993) and infertile males (Mahdi *et al.,* 1999). It may be suggested that an excess of ROS in infertile patients caused the oxidative degradation of seminal plasma lipids resulting in decreased level of lipids in these patients. It is well known that lipids in seminal plasma, apart from serving as energy source for spermatozoa during capacitation and fertilization process (Sugkraroek *et al.,* 1991), also determine their structural integrity (White *et al.,* 1976). As the spermatozoa pass from the caput to the cauda epididymidis, the incorporation of lipids in or on the cell membrane of spermatozoa is an important event in the maturation process (Umapathy *et al.,* 1980). Furthermore, lipid exchange occurs, more or less freely, between spermatozoa and seminal plasma (Huacuja, *et al.,* 1981). Therefore, it may be stated that decrease in the lipid content of seminal plasma in all groups, as reported here, will not only have an adverse effect on the membrane structure required for the occurrence of capacitation and acrosome reaction but may also lead to perturbance of sperm function. Phospholipids, along with cholesterol, play important role in maintaining the cohesiveness of sperm membrane structure and their capability to withstand physical and physiological stress (Philips, 1972). There have been reports that cholesterol: phospholipids ratio influences the structural integrity and fluidity of membranes (White *et al.,* 1976) and increase in this ratio is known to be associated with decrease in fertility (Davis *et al.,* 1979). A decrease in the phospholipid concentration with more or less unaltered cholesterol in seminal plasma, as reported

here, may increase the cholesterol: phospholipid ratio in spermatozoa, which may also affect fertility. Such an increase in this ratio has also been reported earlier in spermatozoa from patients with unexplained infertility (Sugkraroek *et al.,* 1991). Moreover; Tavilani *et al.,* (2007) also reported diminished seminal plasma phospholipid levels in infertile men.

Treatment with *M. pruriens* and *W. somnifera* significantly increased the levels of seminal plasma lipid profiles as compared to pre treatment groups. Both drugs possess potent antioxidant activity (Rajeshwar *et al.,* 2005; Mishra *et al.,* 2000) and we observed that treatment with these natural products in infertile patients inhibited the formation of lipid peroxides; this may have saved the seminal lipids, including triglycerides and phospholipids from breakdown.

Aerobic metabolism of human sperm produces different reactive oxygen species (ROS), which are essential for sperm capacitation, acrosome reaction, and oocyte fusion (Storey, 1997). In order to counteract the toxic effects of ROS, seminal plasma and spermatozoa are well endowed with an array of antioxidant mechanisms. The antioxidant enzymes; catalase, superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase have all been detected in seminal plasma (Mahdi *et al.,* 1999, Ahmad *et al.,* 2009). SODs are metallaoprotein that dismutate the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (McCord and Fridovich, 1988). Thereafter, H_2O_2, which unlike O_2^- can traverse cell membranes easily, must be efficiently converted into H_2O by catalase. GPx, a selenium-containing antioxidative enzyme scavenging system, acts directly as an antioxidant and an inhibitor of lipid peroxidation. GPx could protect the sperm against peroxidative damage (de Lamirande and Gagnon, 1993). GPx also plays an important

role in sperm maturation from the early events up to the onset of fertilization (Vernet *et al.,* 1997). A decrease in GPx activity may lead to reduce fertilizing capacity (Hall *et al.,* 1998). We observed that the activity of seminal GPx was lower in infertile patients than in fertile men (Alkan *et al.,* 1997). Moreover, the activities of other antioxidant enzyme viz. SOD, Catalase and GR were also found reduced in all groups of infertile men and this may be mainly due to elevated levels of oxidative insult (Mahdi *et al.,* 1999; Khosrowbeygi and zarghami, 2007). However, treatment with *M. pruriens and W. somnifera* significantly improved the activity of antioxidant enzymes namely superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase., which may have eventually contributed in reducing the levels of lipid peroxides, spermatozoa ROS and protein carbonyl groups along with increase in the levels of TAC in infertile men. Earlier it has been reported that *M. pruriens and W. somnifera* both have strong antioxidant properties and this might be the reason, in the recovery of antioxidants levels in infertile men (Rajeshwar *et al.,* 2005; Mishra *et al.,* 2000).

Vitamin A is a biological antioxidant which functions as a detoxifying agent, immunopotentiator and immunoactivator (Ames *et al.,* 1993). Similarly, vitamin E (α-tocopherol) also contributes to the body's defense system against lipoprotein oxidation and improvement of sperm motility (Keskes-Ammar *et al.,* 2003). It is well documented that healthy fertile men with normal sperm parameters contain adequate amounts of vitamins A and E (Aitken and Clarkson, 1987). In such men, the antioxidant scavenging system may function normally, or there is low oxidative stress. Conversely, the low seminal levels of vitamins A and E in men with sperm dysfunction may be a reflection of impaired antioxidant activity as a result of increased oxidant stress (Rajasekharan *et al.,* 1995). In the present study we also

observed low levels of vitamins A and E in infertile men and this was thereafter neutralized by treatment with *M. pruriens and W. somnifera.*

Ascorbic acid, a water soluble vitamin, is known to possess potent ROS scavenging activity. In seminal plasma ascorbic acid concentration is 10 fold higher than in serum (Dawson *et al.,* 1987). However, it has been reported that vitamin C levels are significantly reduced in seminal plasma of infertile men (Patel *et al.,* 2009). Significantly reduced levels of ascorbic acid were also observed by us in the present study, and this indicates free radical mediated injury, and this may be either the cause of and /or the result of sperm stress. Treatment with *M. pruriens and W. somnifera* significantly increased the levels of vitamins A, E and C in the seminal plasma of infertile men. *M. pruriens and W. somnifera* contains lipids, antioxidant vitamins and minerals (Pant *et al.,* 1974), which may be helpful as vital supplements and nutrients to maintain the integrity of spermatozoa. Our results indicate the strength of these herbs to protect against vitamin loss.

From the above discussion it is quite clear that reactive oxygen species (ROS)-mediated damage to sperm is a significant contributing pathology in male infertility (Makker *et al.,* 2009 1992; Agarwal *et al.,* 2006). ROS cause infertility by two principal mechanisms. First, ROS damage the sperm membrane which in turn reduces the sperm's motility and ability to fuse with the oocyte. Secondly, ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. In the present study we also observed that spermatozoa of infertile men not only produce significantly high levels of ROS, but they also showed more DNA damage as compared with fertile men. Moreover, our results demonstrated that *M. pruriens* and *W. somnifera* were capable of reducing the ROS levels and seminal lipid peroxides, and this may have been the reason for the reduction in the extent of DNA damage in

the spermatozoa of post-treated men. Furthermore, strong evidence exit to support the fact that elevated levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men (Fraga *et al.,* 1996; Shamsi *et al.,* 2008; Lewis *et al.,* 2008; Tremellen, 2008). The formation of 8-hydroxy-2-deoxyguanosine (8-OhdG) has been considered as a key biomarker for this oxidative DNA damage (Ames *et al.,* 1993). Recently, a significant positive correlation between ROS and DNA fragmentation has been reported (Barroso *et al.,* 2000). Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements (Twigg *et al.,* 1998; Duru *et al.,* 2000).

Endocrinopathy has been reported to be present in up to 23% of men with infertility, but in less than half of patients the etiology is purely endocrine. Circulating levels of various sex hormones have been correlated with semen quality (Meeker *et al.,* 2007). Testosterone plays an important role in spermatogenesis (Islam and Trainer, 1998), while elevated levels of FSH have been correlated with damage to the seminiferous tubules and it has also been suggested as a marker of sertoli cell function and spermatogenesis (Weinbauer and Nieschlag, 1995). Our results demonstrated that LH, T, FSH and PRL hormone levels could be used as markers of semen quality (Ahmad *et al.,* 2009). We observed decreased levels of LH and T and increased levels of FSH and PRL in men with poor semen quality. Moreover, we also found that treatment with *M. pruriens* significantly recovered the levels of testosterone and LH and suppressed FSH and PRL. Furthermore, it was seen that normozoospermic infertile men presented with less perturbed hormone levels and less DNA damage,

despite having high ROS content. The reduced DNA damage may also probably be due to less hormonal imbalance seen in these men. However, oligozoopermic and asthenozoospermic infertile men exhibited more hormonal disbalance and high DNA damage, inspite of oxidative stress levels being similar to that of normozoospermic infertile men. This may indicate the important contribution of sex hormones in protecting against oxidative stress induced DNA damage. Our findings of correction in DNA damage of infertile men by *M. pruriens* is supported by a very recent study by Suresh *et al.,* (2009). They also reported that aged rats exhibited hypo-spermatogenesis, high DNA damage in sperms and had high overall oxidative stress. While treatment with *M. pruriens* lead to the improvement in spermatogenesis, reduction in ROS, spermatozoon DNA damage and overall oxidative stress.

On the basis of available literature and our observations we tried to figure out the possible mechanism of action for both the herbs. *M. pruriens* seeds are rich source of L-DOPA and its metabolites, which include epinephrine and norepinephrine. Though the mode of action of DOPA and catecholamines on human fertility is not yet established, however, this may be linked with the activation of β-adregenic system by increasing the cyclic AMP levels, which in turn regulates the carbohydrate metabolism, lipolysis of fat and functioning of genitourinary and gastrointestinal tracts. There are a few reports that the levels of cAMP in semen of oligozoospermic and azoospermic men are significantly reduced when compared with fertile men (Malachi *et al.,* 1982). Moreover, it is also reported that patients treated with clomiphene citrate exhibit significantly elevated levels of cAMP in their seminal fluid leading to increase in sperm motility. It is well known that spermatogenesis is controlled by the hypothalamus and anterior pituitary working together.

On the basis of afore-stated facts it may be proposed that as *M. pruriens* contains highlevels of L-DOPA (Siddhuraju and Becker, 2001; Prakash *et al.,* 2001), its metabolite, dopamine, may stimulate the hypothalamus and forebrain (Herberg and Rose, 1990) to secrete Gonadotropin-releasing hormone (GnRH) which may further stimulate the anterior pituitary gland to secrete follicule stimulating hormone (FSH) and leutinizing hormone (LH) causing increased synthesis of testosterone by Leydig cells of the testis (Sriraman *et al.,* 2003) and this is elaborated in figure 37 (Ahmad *et al.,* 2008). The action of LH on Leydig cells is brought about by binding of the hormone to specific receptors (Leutinizing hormone receptor, LHR), which are present on the cell membrane and activates cAMP second messenger system (Cooke, 1999). According to Clark *et al.,* (1995), increased levels of cAMP are largely responsible for the increase in steroid production by Leydig cells due to rapid mobililization of cholesterol. Therefore, increased dopamine levels optimize the production of hormones, including testosterone, leading to increased sexual drive and improved performance (Caggiula *et al.,* 1978). We also observed increased plasma levels of testosterone in infertile men following *M. pruriens* therapy. It may be pointed out here that testosterone also possesses potent antioxidative activity (Maiti and Kar, 1997), which might also play an important role in decreasing the lipid peroxide levels in seminal plasma of *M. pruriens* treated infertile men. According to our results, it may be safely concluded that treatment with *M. pruriens* exerted a potent restorative and invigorative effect in all groups of infertile males. Moreover, we also observed that infertile subjects (mainly oligozoospermic and normozoospermic, roughly 30%) were able to achieve pregnancy following treatment with *M. pruriens* (Mahdi, unpublished data).

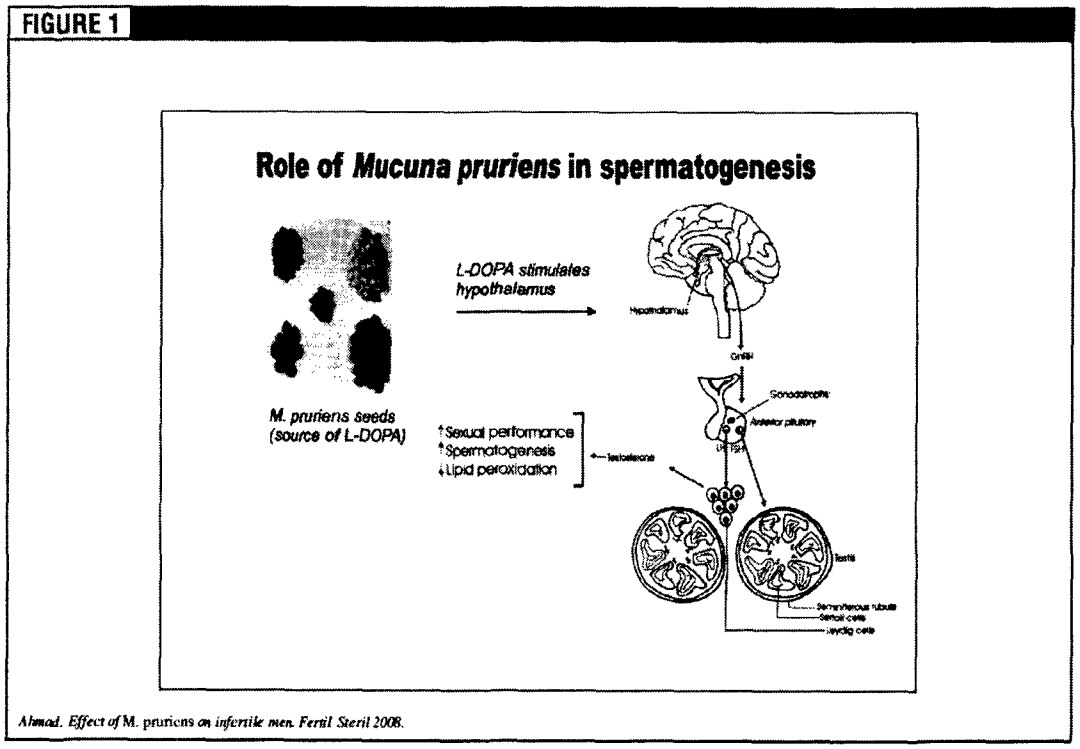


Figure 37: Role of *M. pruriens* in spermatogenesis

The biological basis and exact mechanism of action of *W. somnifera* on infertility is not well known but previous experimental studies showed that treatment with aqueous extracts of *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats (Abdel –Magied *et al.,* 2000). It is reported that *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous tubules (Al-Qarwi *et al.,* 2000). Moreover, *W. somnifera* has also been reported to have several pharmacological effects e.g. antistressor; adaptogenic and cardioprotective properties etc. (Tripathy *et al.,* 1996). The roots of *W. somnifera* contain several alkaloids, withanolides, a few flavanoids and reducing sugars (Umadevi, 1996). More than twenty active constituents have been reported in roots of *W. somnifera* till date including withaferin A, sitoindosides VII–X, withanoside I–VII, choline, beta-sitosterol etc. (Ganzera, *et al.,*2003). The presence of these compounds may be the reason for diverse effects of

W. somnifera on semen properties. However, the spermogenic and /or steroidogenic activity of any of these compounds, if any, has not been explored.

Concluding it may be stated that in the present study we observed significantly high levels of ROS in spermatozoa of infertile men along with elevated levels of lipid peroxides, protein carbonyl groups, decreased total antioxidant capacity and perturbed seminal lipid profiles. We also found suppressed activity of antioxidant enzymes namely SOD, catalase, glutathione reductase and peroxidase in seminal plasma of infertile men along with decreased levels of antioxidants vitamins. Moreover, we observed hormonal imbalance and elevated spermatozoa DNA damage in all groups of infertile men, and this may be the reason for decrease in sperm concentration and motility. Our results showed that *M. pruriens* and *W. somnifera* were able to restore sex hormone levels, reduce oxidative stress and the extent of DNA damage along with the recovery of lipid profiles (Figure 38). Improved spermatogenesis and fertility as a result of *M. pruriens* administration could be attributed to the presence L-DOPA along with a number of other bioactive substances. Similarly, the positive effect of the roots of *W. somnifera* may be due to presence of several alkaloids, Withanolides and flavanoids in it.

Ours is the first report considering oxidative stress, DNA damage and hormonal imbalance along with the protective role of *M. pruriens* and *W. somnifera* in infertile human subjects, and the correlation in between. Although the efficacy of *M. pruriens* in improving male factor fertility was found more in comparison to *W. somnifera,* however, a mixture of the two herbs may be evaluated for better outcome.

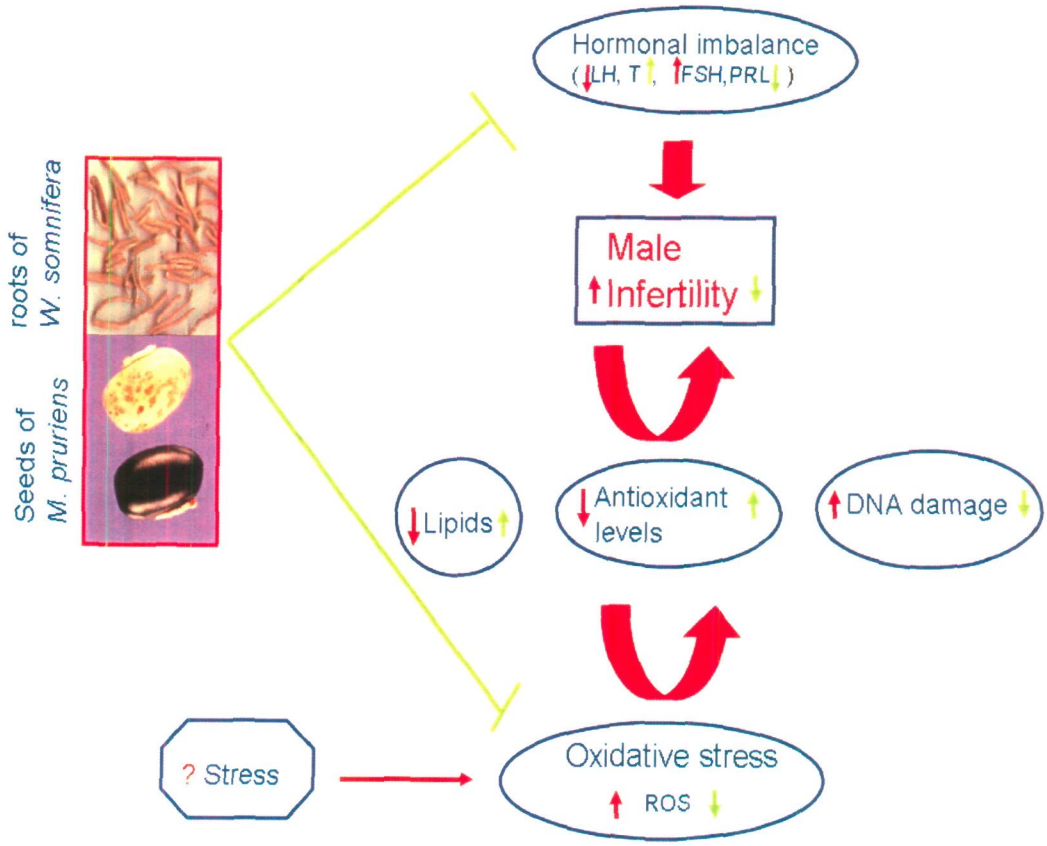


Figure 38: Proposed mechanism of action of *M. pruriens* and *W. somnifera* in preventing DNA damage and infertility. *M. pruriens* and *W. somnifera* restrains hormonal imbalance and oxidative stress to prevent infertility.

Further research on these herbs could focus on fractioning of the herbs and identification of the active constituents. This would not only help in understanding the basis of the treatment offered by these herbs but may also help in understanding the possible causes and novel pathways involved in fertility maintenance in humans. Although the desired effect of the herbs may not be achieved through purified components, but it is still worth giving it a try.

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2. Shukla KK, Mahdi AA, **Ahmad MK**, Shankhwar SN, Jaiswar SP and Tiwari SC. (2007) Effect of *Mucuna pruriens* on stress associated biochemical changes in seminal plasma of infertile males. ***Evidence Based Complementary and Alternative Medicine***, 2007. (online 18th Dec.). **(IF- 2.6)**

3. Shukla, KK, Mahdi, AA, **Ahmad MK**, Shankhwar SN, Singh R, Jaiswar SP. (2008) *Mucuna pruriens* improves male factor fertility by its action on hypothalamus-pituitary-gonadal axis. ***Fertility and Sterility***, online on 29th October. **(IF- 4.2)**

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Effect of *Mucuna pruriens* on semen profile and biochemical parameters in seminal plasma of infertile men

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Objective: To investigate the impact of *Mucuna pruriens* seeds on semen profiles and biochemical levels in seminal plasma of infertile men.
Design: Prospective study.
Setting: Departments of Biochemistry and Obstetrics and Gynecology, King George's Medical University, Lucknow, India.
Patient(s): Sixty normal healthy fertile men (controls) and 60 men undergoing infertility screening.
Intervention(s): High-performance liquid chromatography assay procedure for quantitation of vitamin A and E in seminal plasma. Biochemical parameters in seminal plasma, namely lipids, fructose, and vitamin C, were estimated by standard spectrophotometric procedures.
Main Outcome Measure(s): Before and after the treatment, seminal plasma lipid profile, lipid peroxide, fructose, and antioxidant vitamin levels were measured.
Result(s): Treatment with *M. pruriens* significantly inhibited lipid peroxidation, elevated spermatogenesis, and improved sperm motility. Treatment also recovered the levels of total lipids, triglycerides, cholesterol, phospholipids, and vitamin A, C, and E and corrected fructose in seminal plasma of infertile men.
Conclusion(s): Treatment with *M. pruriens* increased sperm concentration and motility in all the infertile study groups. Oligozoospermic patients recovered sperm concentration significantly, but sperm motility was not restored to normal levels in asthenozoospermic men. Furthermore, in the seminal plasma of all the infertile groups, the levels of lipids, antioxidant vitamins, and corrected fructose were recovered after a decrease in lipid peroxides after treatment. The present study is likely to open new vistas on the possible role of *M. pruriens* seed powder as a restorative and invigorating agent for infertile men. (Fertil Steril® 2008;90:627–35. ©2008 by American Society for Reproductive Medicine.)

Key Words: Male infertility, seminal plasma, *Mucuna pruriens*, lipids, lipid peroxides, antioxidant vitamins

Male infertility is a multifactorial disease process with a number of potential contributing causes. Male factors contribute to almost 50% of cases of infertility; in the remainder, infertility may be due to either a female factor or a combination of male and female factors (1). The World Health Organization (WHO) defines infertility as the inability of a couple to conceive after 1 year of regular, unprotected intercourse (2). A substantial number of couples seek fertility treatment because of poor semen quality, and there is evidence in the literature that male reproductive function seems to have deteriorated considerably in the past 4 to 5 decades. Carlsen et al. (3) observed a significant decline in mean sperm concentration from 113 $\times$ 10^6/mL in 1940 to 66 $\times$ 10^6/mL in 1990, or 0.94 $\times$ 10^6/mL/year.

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Seminal plasma, the liquid component of semen providing a safe surrounding for spermatozoa, is the mixture of secretions from several male accessory glands, including prostate, seminal vesicles, epididymis, and Cowper's gland. It contains a typical combination of proteins, lipids, and carbohydrates, with a minor quantity of a variety of biomolecules and some metals (4). Seminal plasma also contains high concentration of fructose, which is essential for normal sperm metabolism and also serves as a nutrient for spermatozoa during their journey in the female genital tract (5).

Lipids constitute a major component of cellular membranes, and they play an important role in maintaining the structural and functional integrity of the spermatozoa. The seminal plasma is characterized by abundance of cholesterol and phospholipids, mainly sphingomyelins and phosphatidyl ethanolamines, present for the most part in the form of high-density lipoprotein lipids (6). Their secretion is hormone dependent (7). The part played by lipids in capacitation is better understood, and the particular characteristic of lipid composition in seminal fluid allows a successful penetration of ovum by spermatozoa (8). Seminal plasma has a strong

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capacity to maintain a relatively neutral and protective environment for sperm function because it contains a vast array of antioxidants (9). Antioxidant enzymes, namely superoxide dismutase, catalase, and glutathione peroxidase, as well as vitamins A, C, and E, continuously operate to maintain oxidant–antioxidant balance in seminal plasma (10, 11).

The detrimental effects of reactive oxygen species (ROS) on spermatozoa were suggested more than 60 years ago with the demonstration that exposure of sperm to oxygen results in sperm toxicity (12). It has been reported that semen of infertile men contains significantly high levels of ROS, whereas fertile men do not have detectable levels of semen ROS (13). There are several reports that the ROS produced by leukocytes and/or by spermatozoa have deleterious effects on sperm function (14). The half-life of ROS is very short, and it is difficult to detect ROS in semen directly. However, malondialdehyde (MDA), one of the lipid peroxidative end-products produced by ROS when it attacks sperm membrane, can indirectly reflect the damage of sperm (15). Therefore, the determination of MDA concentration in seminal plasma may be taken as one important marker for the diagnosis and treatment of male infertility induced by excessive lipid peroxidation (16, 17).

The susceptibility of human spermatozoa to oxidative stress stems from the abundance of unsaturated fatty acids in the sperm plasma membrane. These unsaturated fatty acids provide fluidity that is necessary for sperm motility and membrane fusion events, such as the acrosome reaction and sperm–egg interaction, both required for natural fertilization. However, the unsaturated nature of molecules predisposes them to free radical attack and ongoing lipid peroxidation throughout the sperm plasma membrane. Once this process has been initiated, accumulation of lipid peroxides occurs on the sperm surface with ensuing sperm dysfunction and sperm death (15, 18); thus lipid peroxidation has a deleterious effect on semen quality (19).

In the Indian system of medicine, the Ayurveda and Unani, some plants and plant products (e.g., *Mucuna pruriens*, *Tinospora cardifolia*, *Asparagus racemosus*, *Withania somnifera*, and *Orchis latifolia*) are characterized by the quality of improvement of endurance against stress, general retardation of the aging process, and improvement of male sexual disorders like psychogenic impotence and unexplained infertility (20). The extracts of different parts of the above plants are of great help in some central mechanism to increase secretion of semen, decrease spermatorrhea, or to act as a restorative and invigorating tonic or aphrodisiac in diseases characterized by weakness or loss of sexual power (21). The seeds of *M. pruriens*, because of the presence of L-3,4 dihydroxyphenyl alanine (L-DOPA), a neurotransmitter precursor, have been used as an effective drug for relief in Parkinson disease and are also given as an aphrodisiac (22). It is reported to be prophylactic against oligospermia and is useful in increasing sperm count as well as ovulation in women (23). Seeds of *M. pruriens* also possess antioxidant, hypoglycemic, lipid-lowering, and neuroprotective properties (24). Its seeds contain the alkaloids mucunine, mucunadine, prurienidine, and nicotine, as well as β-sitosterol, glutathione, lecithin, vernolic acid, and gallic acid. They posses a number of other bioactive substances, including tryptamine, alkylamines, steroids, flavonoids, coumarins, cardenolides, and metals like magnesium, copper, zinc, manganese, and iron (25–27). In view of the above considerations, the present study was undertaken to investigate the impact of *M. pruriens* seeds on semen profiles and the levels of some biochemical parameters in the seminal plasma of infertile men.

MATERIALS AND METHODS

Plant Material

The seeds of the plant *M. pruriens* were purchased from an authorized dealer of Ayurvedic and Unani drug materials in Lucknow, India. The seeds of the plant were identified and authenticated by the National Botanical Research Institute (Lucknow) and were dried under shade and ground to a fine powder with a laboratory grinder. It has been reported that they contain L-DOPA, ascorbic acid, proteins, lipids, alkaloids, and other bioactive compounds, as well as some metals (Table 1) (26).

Patient Selection and Treatment

The Institutional Review Board and Ethics Committee of King George's Medical University, Lucknow, approved this study. Male partners of couples attending the infertility clinic of the Department of Obstetrics and Gynecology, Queen Mary's Hospital, King George's Medical University, Lucknow, were included in the study. Before enrolment in the study each subject's written informed consent was obtained in response to a fully written and verbal explanation of the nature of study. The potential participants, each with infertility persisting longer than 1 year, were examined before the study was conducted. The main inclusion criterion was that infection of accessory glands was ruled out in all subjects. Moreover, subjects having diabetes, hypertension, arthritis, tuberculosis, or human immunodeficiency virus, to be taking drugs, or having other conditions known to influence oxidative stress were excluded. Complete physical, biochemical, and semen examinations were performed as the screening test. Additionally, medical histories of patients and their female partners were recorded. The age of the subjects was 30–40 years. The study included two groups of 60 subjects each: the control group and the study group (consisting of 20 subjects each in three subgroups: normospermic, oligospermic, and asthenospermic).

The control group comprised age-matched healthy men who had previously initiated at least one pregnancy and exhibited a normal semen profile ($>20 \times 10^6$ spermatozoa/mL, >40% motility, and >40% normal morphology). The study group comprised [1] normozoospermic infertile men (n = 20), having a normal semen profile (defined as in the control

TABLE 1

The major biologically active components of *Mucuna pruriens* seeds.

Constituents*	% wt/wt	Metals	mg per 100 g seed flour
L-DOPA	3.6–4.2	Magnesium	174.9–387.6
Alkaloids (mucunine, mucunadine, prurienidine and nicotine)	0.53	Zinc	5.0–10.9
Ascorbic acid	4.78	Iron	10.8–15.0
Total protein (albumins, globulins, prolamins, glutelins) and amines (tryptamine, alkylamine)	20.2–29.3	Copper	0.9–2.2
Total lipids (β-sitosterol, lecithin)	6.3–7.4	Manganese	3.9–4.3
Total dietary fiber	8.7–10.5	Sodium	43.1–150.1
Ash	3.3–5.5	Potassium	778.1–1846
Energy level	1,562–1,597 kJ 100 g^{-1} DM	Phosphorus	325.8–592.0

Note: L-DOPA = L-3,4 dihydroxyphenyl alanine.
Data from references 25–27.
* The seeds also contain flavonoids, coumarins, and cardenolides in traces.

Ahmad. Effect of M. pruriens on infertile men. Fertil Steril 2008.

group) and infertility of unknown etiology and whose infertile female partners had undergone extensive infertility evaluation without showing a detectable gynecologic abnormality; [2] oligozoospermic infertile men (n = 20), having a sperm count $<20 \times 10^6$/mL, motility >40%, and >40% normal morphology; and [3] asthenozoospermic infertile men (n = 20), having a sperm count $>20 \times 10^6$/mL, motility <40%, and >40% normal morphology. After examination of the semen samples, infertile men were prescribed *M. pruriens* seed powder (5 g/d) orally for 3 months with milk. This dosing schedule was as reported earlier by Singh (28). Semen samples were collected twice: before administration of the drug and after 3 months' treatment with the drug. All semen profiles were evaluated within 1 hour of specimen collection and biochemical parameters within 2 days.

Semen Collection, Seminal Plasma Preparation, and Assessment

Semen samples were collected into sterile plastic containers by masturbation after 3 to 4 days of abstinence. Semen volume was recorded after liquefaction of semen; an aliquot was taken to assess sperm motility and count. A semen profile was constructed with the procedures described by WHO (29). The semen samples after liquefaction were centrifuged at $1,200 \times g$ for 20 minutes for separation of seminal plasma. The supernatant (seminal plasma) was centrifuged at $10,000 \times g$ for 30 minutes to eliminate all possible contaminating cells. Seminal plasma was taken for different biochemical assessments. The supernatant was quickly frozen and stored at −20°C until analyzed. Seminal plasma lipid peroxides were estimated according to the method of Ohkawa et al. (30), with modifications as described by Sanocka et al. (31) using thiobarbituric acid. Vitamins A and E were measured by high-performance liquid chromatography (HPLC) as per the modified method of Omu et al. (32). Briefly α-tocopherol acetate and retinol acetate were pipetted into an Eppendorf tube. Into this, seminal plasma was added and vortex mixed; hexane extract of vitamins A and E was aspirated out in a glass tube, dried under nitrogen stream, and dissolved into methanol. Finally, this preparation was injected into HPLC fitted with a reverse phase C-18 stainless steel column. The vitamins were eluted with methanol at the flow rate of 1.5 mL/min for 15 minutes. The peak heights and the curve areas of vitamins A and E and their acetate were measured to calculate the amount of these vitamins in seminal plasma in an ultraviolet detector with 292-nm filters. Ascorbic acid and fructose levels were estimated as described by Beutler (33) and Gavella (34). Corrected seminal fructose values were deduced by multiplying the logarithm of sperm concentration and seminal plasma fructose concentration (35). Seminal plasma lipids were extracted by the method of Folch et al. (36). The extract was used for estimation of total lipids (37), cholesterol (38), and phospholipids (39). Triglycerides were measured using an enzymatic assay kit (40).

Statistical Analysis

The four independent groups were control, pretreated normozoospermic, pretreated oligozoospermic, and pretreated asthenozoospermic, and they were compared together by one-way analysis of variance followed by Dunnett's test. A paired t test was used to analyze the significance of the mean difference between pre- and posttreatment infertile groups. Relative associations among parameters were determined by Pearson's correlation coefficient. All hypothesis testing was two tailed. The results are expressed as mean ± SD, and $P<.05$ was considered statistically significant. The statistical analysis was carried out with commercial software (INSTAT 3.0; GraphPad Software, San Diego, CA).

RESULTS

Semen Profile

The semen profiles of the fertile (control) group and the pre- and post-*M. pruriens*-treated infertile groups are shown in Table 2. The values in fertile men were as follows: semen volume 2.95 ± 0.39 mL, sperm concentration 86.55 ± 12.68 $\times 10^6$/mL, sperm count 254.73 ± 47.83 $\times 10^6$ per ejaculate, and motility 77.58% ± 9.22%. These parameters were found to be significantly decreased in all infertile patients compared with controls. Treatment of these infertile men with *M. pruriens* seed powder (5 mg/d) for 3 months showed a significant reversal of the above parameters. Treatment with *M. pruriens* significantly increased the sperm concentration in normozoospermic (55.33 ± 13.58 $\times 10^6$/mL vs. 91.96 ± 7.5 $\times$ 10^6/mL; $P<.01$), oligozoospermic (8.7 ± 1.47 $\times$ 10^6/mL vs. 52.78 ± 6.56 $\times$ 10^6/mL; $P<.01$), and asthenozoospermic men (46.96 ± 6.77 $\times$ 10^6/mL vs. 68.69 ± 4.15 $\times$ 10^6/mL; $P<.01$) compared with the pretreatment groups. Furthermore, motility was also significantly increased in normozoospermic (60.5% ± 8.87% vs. 73.23% ± 6.93%; $P<.01$), oligozoospermic (59.5% ± 8.87% vs. 68.25% ± 7.3%; $P<.01$), and asthenozoospermic men (15.15% ± 2.27% vs. 22.85% ± 4.54%; $P<.01$) after 3 months' treatment with *M. pruriens*. The semen volume was significantly increased ($P<.01$), as was the sperm count per ejaculate ($P<.01$), in normozoospermic, oligozoospermic, and asthenozoospermic men compared with pretreatment levels.

Lipid Profile

The level of lipid peroxides (LPO) in the seminal plasma of fertile men was 1.85 ± 0.11 nmol MDA/mL. This parameter was increased in the seminal plasma of normozoospermic (3.57 ± 0.26 nmol MDA/mL; $P<.01$), oligozoospermic (2.5 ± 0.15 nmol MDA/mL; $P<.01$), and asthenozoospermic men (3.3 ± 0.23 nmol MDA/mL; $P<.01$) (Table 3). A significant reversal in the level of LPO was observed in normozoospermic (2.14 ± 0.19 nmol MDA/mL; $P<.01$), oligozoospermic (1.93 ± 0.15 nmol MDA/mL; $P<.01$), and asthenozoospermic men (2.21 ± 0.18 nmol MDA/mL; $P<.01$) after treatment with *M. pruriens*. The levels of total lipids, cholesterol, triglycerides, and phospholipids in the seminal plasma of fertile men were 376.08 ± 37.23 mg/dL, 59.49 ± 7.25 mg/dL, 62.97 ± 6.94 mg/dL, and 182.63 ± 20.48 mg/dL, respectively. These levels were significantly reduced in all groups of infertile men compared with controls. Treatment with *M. pruriens* significantly recovered the level of total lipids in normozoospermic, oligozoospermic, and asthenozoospermic men ($P<.01$) compared with pretreatment levels. Similarly, *M. pruriens* treatment also significantly recovered the seminal plasma levels of cholesterol, triglycerides, and phospholipids in normozoospermic ($P<.01$), oligozoospermic ($P<.01$), and asthenozoospermic men ($P<.01$) compared with pretreatment levels.

Corrected Fructose and Antioxidant Vitamins

Table 4 shows that the levels of corrected seminal fructose and vitamins A, E, and C in the seminal plasma of infertile men were 3.58 ± 0.38 mg/mL, 27.72 ± 4.41 μg/dL, 0.141 ± 0.012 mg/dL, and 5.97 ± 0.67 mg/dL, respectively. These parameters were decreased in all groups of infertile men compared with controls. Treatment with *M. pruriens* recovered the levels of corrected seminal fructose in normozoospermic, oligozoospermic, and asthenozoospermic men ($P<.01$) compared with pretreatment levels. Furthermore, after treatment the levels of vitamins A, E, and C were also restored in normozoospermic ($P<.01$), oligozoospermic ($P<.01$), and

TABLE 2

Effect of *Mucuna pruriens* on semen profile of infertile men.

Group	Treatments	Semen volume (mL)	Sperm concentration ($\times 10^6$/mL)	Sperm count ($\times 10^6$ per ejaculate)	Motility
Control (n = 60)		2.95 ± 0.39	86.55 ± 12.68	254.73 ± 47.83	77.58 ± 9.22
Normozoospermic (n = 20)	Pretreatment[a]	2.07 ± 0.38	55.33 ± 13.58	114.82 ± 34.89	60.5 ± 8.87
	Posttreatment[b]	2.8 ± 0.3	91.96 ± 7.50	258.21 ± 39. 11	73.25 ± 6.93
Oligozoospermic (n = 20)	Pretreatment[a]	1.98 ± 0.3	8.7 ± 1.47	17.29 ± 4.0	59.5 ± 8.87
	Posttreatment[b]	2.28 ± 0.28	52.78 ± 6.56	119.96 ± 15.64	68.25 ± 7.3
Asthenozoospermic (n = 20)	Pretreatment[a]	2.33 ± 0.23	46.96 ± 6.77	109.03 ± 16.96	15.15 ± 2.27
	Posttreatment[b]	2.38 ± 0.22	68.69 ± 4.15	163.45 ± 18.31	22.85 ± 4.54

[a] $P<.01$ for all parameters compared with control (Dunnett test).
[b] $P<.01$ for all parameters compared with pretreatment (paired *t* test).

Ahmad. Effect of M. pruriens on infertile men. Fertil Steril 2008.

TABLE 3

Effect of *M. pruriens* on lipid profile in seminal plasma of infertile men.

Group	Treatments	Lipid peroxides (nmol MDA/mL)	Total lipids (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Phospholipids (mg/dL)
Control (n = 60)		1.85 ± 0.11	376.08 ± 37.23	59.49 ± 7.25	62.97 ± 6.94	182.63 ± 20.48
Normozoospermic (n = 20)	Pretreatment[a]	3.57 ± 0.26	278.08 ± 28.43	46.85 ± 5.80	53.31 ± 6.84	144.91 ± 16.59
	Posttreatment[b]	2.14 ± 0.19	357.21 ± 29.01	56.01 ± 4.65	59.62 ± 8.43	169.44 ± 16.88
Oligozoospermic (n = 20)	Pretreatment[a]	2.5 ± 0.15	261.3 ± 31.1	51.5 ± 8.44	40.83 ± 6.75	126.81 ± 18.80
	Posttreatment[b]	1.93 ± 0.15	345.8 ± 29.13	55.91 ± 7.48	55.29 ± 8.54	158.16 ± 19.64
Asthenozoospermic (n = 20)	Pretreatment[a]	3.3 ± 0.23	237.13 ± 35.2	50.15 ± 7.24	42.45 ± 7.54	136.31 ± 15.39
	Posttreatment[b]	2.21 ± 0.18	302.26 ± 41.38	52.25 ± 6.70	51.23 ± 7.86	161.13 ± 17.41

Note: MDA = malondialdehyde.

[a] $P<.01$ for all parameters compared with control (Dunnett test).

[b] $P<.01$ for all parameters compared with pretreatment (paired t test).

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asthenozoospermic men ($P<.01$) compared with pretreatment levels.

Table 5 shows that the correlations between different pre- and posttreatment parameters in infertile men were positive and significant, except semen volume and sperm concentration in oligozoospermic men, total lipids in normozoospermic men, and vitamin E in asthenozoospermic men, which showed the opposite association.

DISCUSSION

The results of present study demonstrate that treatment of infertile subjects with *M. pruriens* for 3 months significantly improved sperm concentration and motility. It has been reported that *M. pruriens* helps in some central mechanism to increase secretion of semen, decrease spermatorrhea, and act as a restorative and invigorating tonic and aphrodisiac in diseases characterized by weakness or loss of sexual power (21). The biological basis and exact mechanism of action of *M. pruriens* on infertility is not well known. However, the beneficial effect may be attributed to its antioxidant and neurostimulatory properties (27). *Mucuna pruriens* is reported to contain many bioactive constituents, including alkaloids, coumarins, flavonoids, and alkylamines, which may play an important role in increasing the antioxidant capacity of treated men. There are also reports that the methanol extract of *M. pruriens* seeds has strong antioxidant activity, because it inhibits 1,1-diphenyl-2-picryl-hydrazyl and hydroxyl radical, and that it also has nitric oxide and superoxide anion scavenging and hydrogen peroxide decomposing and reducing power (41).

Furthermore, we found that treatment with *M. pruriens* significantly decreased the lipid peroxide levels in infertile men. There have been reports that lipid peroxide levels are significantly elevated in the seminal plasma of infertile men (42). It is known that lipid peroxidation is a free radical–mediated phenomenon and that the lipids in spermatozoa are susceptible to peroxidation (13). Aitken et al. (43) have demonstrated the involvement of lipid peroxidation in the pathophysiology of male infertility.

Moreover, our results also showed that treatment with *M. pruriens* significantly increased the levels of seminal plasma lipids, especially phospholipids, and restored the ratio of cholesterol to phospholipids. Recovery of seminal plasma lipids, including cholesterol, triglycerides, and phospholipids, may be due to the inhibition of lipid peroxidation, as evidenced by lower LPO levels, after *M. pruriens* treatment. This may also help in minimizing oxidative stress-mediated cellular toxicity.

There are also reports that *M. pruriens* seeds contain substantial amount of metals (Table 1), which may provide vital supplements and nutrients. Aydemir et al. (44) reported that copper and iron play an important role in spermatogenesis and fertility. However, their levels are also elevated in infertile men. It is likely that they may be responsible for ROS generation. Copper and iron can promote ROS formation

TABLE 4

Effect of *M. pruriens* on seminal plasma levels of corrected fructose and antioxidant vitamins in infertile men.

Group	Treatments	Corrected fructose (mg/mL)	Vitamin A (μg/dL)	Vitamin E (mg/dL)	Vitamin C (mg/dL)
Control (n = 60)		3.58 ± 0.38	27.72 ± 4.41	0.141 ± 0.012	5.97 ± 0.67
Normozoospermic (n = 20)	Pretreatment[a]	2.63 ± 0.34	18.76 ± 2.33	0.115 ± 0.011	4.48 ± 0.35
	Posttreatment[b]	3.4 ± 0.47	23.04 ± 2.53	0.142 ± 0.009	5.49 ± 0.35
Oligozoospermic (n = 20)	Pretreatment[a]	2.27 ± 0.31	17.33 ± 2.97	0.099 ± 0.014	5.34 ± 1.01
	Posttreatment[b]	3.03 ± 0.3	20.13 ± 3.01	0.131 ± 0.014	5.74 ± 0.9
Asthenozoospermic (n = 20)	Pretreatment[a]	2.27 ± 0.4	15.74 ± 2.43	0.089 ± 0.012	5.12 ± 0.78
	Posttreatment[b]	2.79 ± 0.34	19.32 ± 2.28	0.136 ± 0.019	5.78 ± 0.87

[a] $P < .01$ for all parameters compared with control (Dunnett test).
[b] $P < .01$ for all parameters compared with pretreatment (paired *t* test).

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by catalyzing the reaction between superoxide ion and hydrogen peroxide, producing the hydroxyl radical. Moreover, copper may also promote crosslinking of proteins. The levels of copper and iron in *M. pruriens* are 0.045–0.11 mg and 0.54–0.75 mg per 5 g seed flour, respectively (25), which is not very high. Additionally, *M. pruriens* contains high amounts of antioxidants, which may prevent ROS generation and further augment the antioxidative system. This is evident from reduction in LPO levels in treated infertile men. Furthermore, *M. pruriens* seeds contain magnesium and zinc (8.74–19.38 mg and 0.25–0.54 mg per 5 g seed flour, respectively), which are important for spermatogenesis (45) and also serve as cofactors for many metalloenzymes (46).

Interestingly, we observed that seminal plasma levels of vitamins A, C, and E were significantly increased in infertile men after treatment with *M. pruriens*. This might have contributed to the improvement in sperm concentration and motility and the lowering of lipid peroxide levels in the seminal plasma of infertile men. Vitamins A, C, and E are biological antioxidants that function as detoxifying agents, immunopotentiators, and immunoactivators (47).

In the present study, the corrected seminal fructose levels were deduced as log10 sperm concentration multiplied by seminal fructose concentration. This has been shown to be a better marker of seminal vesicle function than the simple

TABLE 5

Pearson correlation between pre- and posttreatment parameters in infertile men.

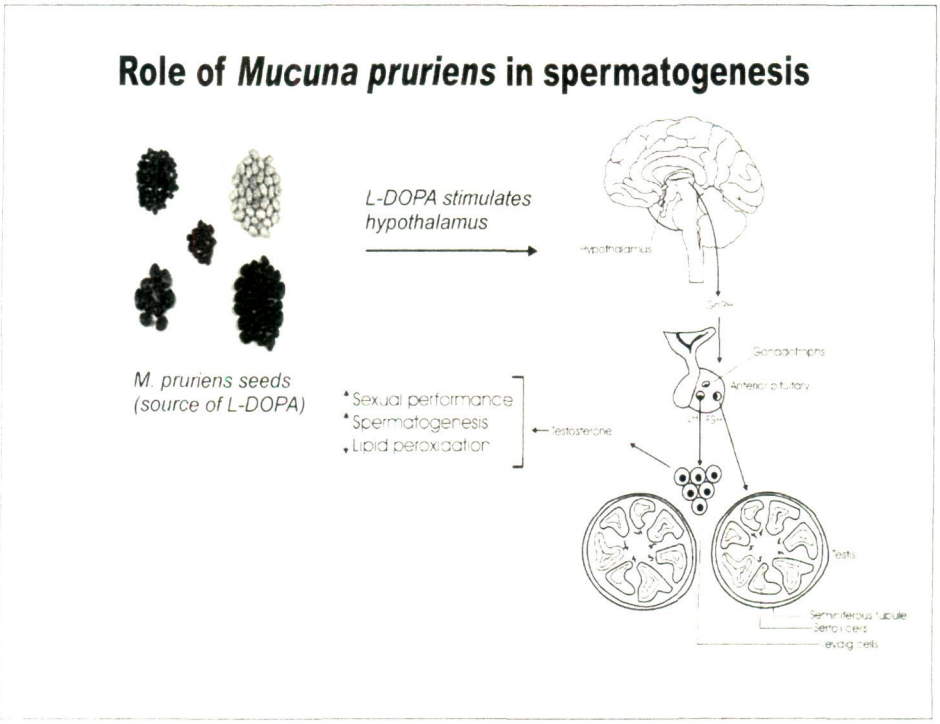
Parameter	Normozoospermic	Oligozoospermic	Asthenozoospermic
Semen volume	0.79[a]	0.36 (NS)	0.95[a]
Sperm concentration	0.71[a]	−0.14 (NS)	0.64[a]
Sperm count per ejaculate	0.70[a]	0.43[b]	0.71[a]
Motility	0.80[a]	0.77[a]	0.71[a]
Lipid peroxides	0.72[a]	0.23[a]	0.84[a]
Total lipids	0.05 (NS)	0.93[a]	0.90[a]
Cholesterol	0.91[a]	0.87[a]	0.99[a]
Triglyceride	0.70[a]	0.82[a]	0.96[a]
Phospholipids	0.92[a]	0.95[a]	0.80[a]
Corrected fructose	0.83[a]	0.59[a]	0.86[a]
Vitamin A	0.81[a]	0.93[a]	0.84[a]
Vitamin E	0.83[a]	0.86[a]	0.36 (NS)
Vitamin C	0.54[a]	0.92[a]	0.73[a]

Note: NS = not significant ($P > .05$).
[a] Highly significant ($P < .01$).
[b] Significant ($P < .05$).

Ahmad. Effect of M. pruriens on infertile men. Fertil Steril 2008.

FIGURE 1

Role of *Mucuna Pruriens* in spermatogenesis.



Ahmad. Effect of M. pruriens *on infertile men. Fertil Steril 2008.*

measurement of the seminal fructose concentration (48). Corrected fructose is correlated with sperm motility in men with normal sperm motility, whereas seminal fructose is not. The recovered values of seminal corrected fructose after treatment with *M. pruriens* were coupled with an increase in sperm concentration and motility in normozoospermic and oligozoospermic men. In the case of asthenozoospermic men, the recovery of corrected fructose did not exhibited significant correlation with sperm motility.

Mucuna pruriens seeds are a rich source of L-DOPA and its metabolites, which include epinephrine and norepinephrine. Though the mode of action of DOPA and catecholamines on human infertility is not yet established, this may be linked with the activation of the β-adrenergic system by increasing the cyclic adenosine monophosphate (cAMP) levels, which in turn regulates the carbohydrate metabolism, lipolysis of fat, and functioning of genitourinary and gastrointestinal tracts. There are a few reports that the levels of cAMP in the semen of oligozoospermic and azoospermic men are significantly reduced compared with levels in fertile men (49). Moreover, it is also reported that patients treated with clomiphene citrate exhibit significantly elevated levels of cAMP in their seminal fluid, leading to an increase in sperm motility. It is well known that spermatogenesis is controlled by the hypothalamus and anterior pituitary working together.

On the basis of aforementioned facts, it may be proposed that because *M. pruriens* contains high levels of L-DOPA (26, 50), its metabolite, dopamine, may stimulate the hypothalamus and forebrain (51) to secrete GnRH, which may further stimulate the anterior pituitary gland to secrete FSH and LH, causing increased synthesis of T by Leydig cells of the testis (52). This is elaborated in Figure 1. The action of LH on Leydig cells is brought about by binding of the hormone to specific receptors (LH receptors) that are present on the cell membrane and activate the cAMP second messenger system (53). According to Clark et al. (54), increased levels of cAMP are largely responsible for the increase in steroid production by Leydig cells, owing to rapid mobilization of cholesterol. Therefore, increased dopamine levels optimize the production of hormones, including T, leading to increased sexual drive and improved performance (55). We also observed increased plasma levels of T in infertile men after *M. pruriens* therapy (Mahdi, unpublished observations). Testosterone also possesses potent antioxidative activity (56), which might also play an important role in decreasing the lipid peroxide levels in the seminal plasma of *M. pruriens*–treated infertile men.

According to our results, it may be safely concluded that treatment with *M. pruriens* exerted a potent restorative and invigorating effect in all groups of infertile men. We also observed that infertile subjects (mainly oligozoospermic and normozoospermic men, roughly 30%) were able to achieve pregnancy after treatment with *M. pruriens* (Mahdi, unpublished data). In light of the encouraging results of the present study, there is a need for further in-depth studies to

investigate the role of *M. pruriens* and its biologically active compounds in increasing the fertilizing potential in infertile men.

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Withania somnifera improves semen quality by regulating reproductive hormone levels and oxidative stress in seminal plasma of infertile males

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Objective: To investigate the impact of *Withania somnifera* roots on semen profile, oxidative biomarkers, and reproductive hormone levels of infertile men.

Design: Prospective study.

Setting: Departments of Biochemistry and Urology, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India.

Patient(s): Seventy-five normal healthy fertile men (control subjects) and 75 men undergoing infertility screening.

Intervention(s): High-performance liquid chromatography assay procedure for quantization of vitamin A and E in seminal plasma. Biochemical parameters in seminal plasma were estimated by standard spectrophotometric procedures. Estimation of T, LH, FSH, and PRL in blood serum by RIA methods.

Main Outcome Measures(s): Before and after the treatment, seminal plasma biochemical parameters, antioxidant vitamins, and serum T, LH, FSH, and PRL levels were measured.

Result(s): *Withania somnifera* inhibited lipid peroxidation and protein carbonyl content and improved sperm count and motility. Treatment of infertile men recovered the seminal plasma levels of antioxidant enzymes and vitamins A, C, and E and corrected fructose. Moreover, treatment also significantly increased serum T and LH and reduced the levels of FSH and PRL.

Conclusion(s): The treatment with *W. somnifera* effectively reduced oxidative stress, as assessed by decreased levels of various oxidants and improved level of diverse antioxidants. Moreover, the levels of T, LH, FSH and PRL, good indicators of semen quality, were also reversed in infertile subjects after treatment with the herbal preparation. (Fertil Steril® 2009;■:■–■. ©2009 by American Society for Reproductive Medicine.)

Key Words: *Withania somnifera,* male infertility, alternative therapy, reproductive hormone, oxidative stress, antioxidant

Infertility may be defined as failure to conceive by a couple after 12 months of unprotected sexual intercourse (1). Infertility affects 15% of all couples, and approximately 50% of these have an abnormality detectable in the male partner as the cause of infertility. Specific and directed treatment for male infertility is not available owing to the unexplained and heterogeneous nature of the disorders (2). Under such circumstances, only assisted reproductive technologies are of some help. However, these treatments are expensive and inaccessible to all. The lack of available specific therapies for men with infertility demands the exploration of alternative therapies. Given the lack of knowledge about etiologic factors, a nondirected but general therapy may yield good results in a subcategory of patients. The rationale for the use of these therapies is based on the speculation that some forms of male infertility are caused by oxidative insult and hormonal imbalance, and the use of alternative therapies may improve male fertility potential and semen quality (3). The latter is also supported by our previous studies (4–6).

Aerobic metabolism of human sperm produces different reactive oxygen species (ROS), which are essential for sperm capacitation, acrosome reaction, and oocyte fusion (7). To counteract the toxic effects of ROS, seminal plasma and spermatozoa are well endowed with an array of antioxidant mechanisms. The antioxidant enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase, and glutathione reductase have all been detected in seminal plasma (8). In addition, semen contains high concentration of thiol groups, ascorbic acid and uric acid, as well as less substantial

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amounts of glutathione and α-tocopherol (9). Spermatozoa themselves also possess high concentration of thiol groups, as well as smaller amounts of ascorbic acid, α-tocopherol, uric acid, and glutathione (10). However, uncontrolled and excessive production of ROS may result in seminal oxidative stress (11). The imbalance between ROS production and ROS degradation has been hypothesized as a cause of oxidative stress in semen, with peroxidative injury to the sperm membrane and a consequent impairment of the related functional properties, such as sperm motility and morphology. (12). Growing evidence suggests that such seminal oxidative stress is involved in many cases of idiopathic male factor infertility (11).

In the Ayurveda and Unani systems of medicine practiced in India, several plants and plant products have been documented to fight against stress, impotence, infertility, and the aging process (13). *Withania somnifera*, also known as Indian ginseng, has been described in folk medicine as an aphrodisiac and geriatric tonic. Different investigators have reported that *W. somnifera* possesses antiserotogenic, anticancer, and anabolic activity and is beneficial in the treatment of arthritis, geriatric problems, stress, and male sexual dysfunction. It also possesses adaptogenic, cardiotropic, cardioprotective, and anticoagulant properties (14). *W. somnifera* has been shown to inhibit lipid peroxidation in stress-induced animals (15). Earlier studies have shown that aqueous extract of this plant elicits changes in pituitary gonadotropins coupled with an enhancement in epididymal sperm pattern in adult male rats and folliculogenesis in immature female rats (16). *W. somnifera* induced testicular development and spermatogenesis in immature Wistar rats by directly affecting the seminiferous tubules (17). In view of the above considerations, the present study was undertaken to investigate the impact of *W. somnifera* on semen profile, oxidative biomarkers, and reproductive hormone levels of infertile men.

MATERIALS AND METHODS

Plant Materials

The roots of *W. somnifera* were procured from the Central Council for Research in Unani Medicine, New Delhi. The roots were dried under shade and made to fine powder using a laboratory grinder.

Study Design

The Institutional Review Board and Ethics Committee of Chhatrapati Shahuji Maharaj (CSM) Medical University, Lucknow, approved this study. The study was conducted between February 2007 and August 2008, and the study population included normal healthy and fertile men (n = 75) and infertile patients (n = 75), aged 25–40 years, recruited from the Outpatient Department of Urology, CSM Medical University. The control group comprised age-matched healthy men who had previously initiated at least one pregnancy and exhibited normal semen profile (sperm count >20 × 10^6/mL, motility >40%, and >40% normal morphology). Normozoospermic infertile men (n = 25) had normal semen profile (defined as in the control group) and infertility of unknown etiology. Oligozoospermic infertile men (n = 25) had a sperm count of <20 × 10^6/mL, motility >40%, and >40% normal morphology. Asthenozoospermic infertile men (n = 25) had a sperm count of >20 × 10^6/mL, motility <40%, and >40% normal morphology. Before enrollment in the study, each subject's informed written consent was obtained in response to a fully written and verbal explanation of the nature of the study. The potential participants, each with infertility persisting longer than 1 year, were carefully examined. As an inclusion criterion, the infection of accessory glands was ruled out in all subjects. Moreover, subjects having diabetes, hypertension, arthritis, tuberculosis, or human immunodeficiency, those on drugs, and those having other conditions known to influence oxidative stress were excluded. Complete physical, biochemical, and semen examinations were performed as the screening tests. Additionally, medical histories of patients and their female partners were recorded. Infertile cases where a problem could be diagnosed in the female partner were excluded from the study. All subjects were instructed to continue normal diets without switching to dietary supplements during the course of treatment. To further ensure this, the details of the diet were taken from the patients at monthly intervals.

Treatment

Infertile men were prescribed *W. somnifera* root powder (5 g/day) orally for 3 months with milk. This dosing schedule was as reported earlier by Singh (18).

Sample Collection and Preparation

Semen samples were collected into sterile plastic containers by masturbation after 3–4 days of abstinence and allowed to liquefy for 30 minutes. Semen volume was recorded after liquefaction; an aliquot was taken to assess sperm motility and count. Semen profile was constructed with the procedures described by the World Health Organization (19). Liquefied semen samples were centrifuged at 1,200*g* for 20 minutes for separation of seminal plasma. The supernatant (seminal plasma) was centrifuged at 10,000*g* for 30 minutes to eliminate all possible contaminating cells. Seminal plasma was quickly frozen and stored at −20°C until the assessment of different biochemical parameters. Venous blood samples were drawn between 8 a.m. and 10 a.m. and centrifuged at 3,000*g* at 4°C for 10 minutes and serum was aspirated out for hormone assays.

Hormonal Assays

Serum T, LH, FSH, and PRL were measured by a double-antibody RIA method using Gamma Counter (Stratec Biomedical System, Birkenfeld, Germany) (20). Intra- and inter-assay coefficients of variation in T, LH, FSH, and PRL were 10.0%, 14.0%, 8.5%, and 12.5%, respectively. Receiver's

operating characteristic (ROC) curves were created for the hormone values to understand their diagnostic value.

Biochemical Assay

Seminal plasma lipid peroxides were estimated according to the method of Ohkawa et al. (21), with modifications as described by Sanocka et al. (22), and protein carbonyl group by the method of Levine et al. (23). Ascorbic acid and fructose levels were estimated as described by Butler (24) and Gavella (25). Corrected seminal fructose values were deduced by multiplying the logarithm of sperm concentration and seminal plasma fructose concentration (26). Seminal plasma SOD and catalase activities were measured according to McCord and Fridovich (27) and Aebi (28), respectively, and reduced glutathione level was estimated as described by Hissin and Hilf (29).

Vitamins A and E were measured by high-performance liquid chromatography (HPLC) per the modified method of Omu et al. (30). Briefly α-tocopherol acetate and retinol acetate were pipetted into an Eppendorf tube. To this, seminal plasma was added and vortex mixed. Hexane extract of vitamins A and E was aspirated out in a glass tube, dried under nitrogen stream, and dissolved into methanol. Finally, this preparation was injected into HPLC fitted with a reverse-phase C-18 stainless steel column. The vitamins were eluted with methanol at a flow rate of 1.5 mL/min for 15 minutes. The peak heights and the curve areas of vitamins A, E and their acetates were measured to calculate the amount of these vitamins in seminal plasma in an ultraviolet detector with 292-nm filters.

Statistical Analysis

The four independent groups—control, pretreated normozoospermic, pretreated oligozoospermic, and pretreated asthenozoospermic—were compared by one-way analysis of variance followed by Dunnett test. A paired *t* test was used to analyze the significance of mean difference between pre- and posttreatment infertile groups. All hypothesis testing was two tailed. The results were expressed as mean ± SD, and $P<.05$ was considered to be statistically significant. The statistical analyses were carried out with commercial software (Instat 3.0; GraphPad Software, San Diego, CA). The ROC curves were constructed using SPSS (Version 12; SPSS, Chicago, IL), and cutoff values for each of the four hormones were determined that could be used as indicators of infertility. Low values of LH and T and high values of FSH and PRL were assumed to be better indicators of infertility for construction of ROC curves.

RESULTS

Semen Profile

The semen profiles of the fertile (control) group and the pre– and post–*W. somnifera*–treated infertile groups are shown in Table 1. Sperm concentration increased significantly in normozoospermic, oligozoospermic, and asthenozoospermic men compared with the pretreatment parameters. Motility was also significantly improved in all groups of infertile individuals (Table 1) after treatment, but the increase was less than optimal in asthenozoospermic infertile men. The semen volume was significantly increased ($P<.01$) in normozoospermic and oligozoospermic, whereas in asthenozoospermic the change was not significant. Sperm count per ejaculate was found to be significantly improved ($P<.01$) upon treatment.

Oxidative Biomarkers

The level of lipid peroxides (LPO) and protein carbonyl groups in the seminal plasma of fertile men were 1.74 ± 0.19 nmol Malondialdehyde (MDA)/mL and 2.07 ± 0.37 nmol/mg protein, respectively. These parameters were significantly elevated in the seminal plasma of all the infertile groups (Table 2). Upon treatment, significant reversal in the level of LPO and protein carbonyl groups were observed in normozoospermic, oligozoospermic, and asthenozoospermic men ($P<.01$). The SOD activity in seminal plasma of the control group was 8.17 ± 0.71 U/mg protein. However, this enzyme was found to be significantly suppressed in the groups of infertile men. Treatment significantly enhanced the activity of SOD in normozoospermic, oligozoospermic, and asthenozoospermic men compared with pretreatment parameters. Similarly, treatment significantly recovered the seminal plasma activity of catalase in all the infertile groups ($P<.01$). Reduced glutathione also improved significantly upon treatment ($P<.01$).

Antioxidant Vitamins and Corrected Fructose

The levels of vitamins A, E, and C and corrected seminal fructose in the seminal plasma of fertile men were 28.61 ± 4.43 μg/dL, 0.143 ± 0.012 mg/dL, 5.64 ± 0.71 mg/dL, and 3.63 ± 0.34 mg/mL, respectively (Table 3). These parameters were suboptimal in all groups of infertile men. Treatment recovered the levels of vitamins A, E, and C in normozoospermic ($P<.01$), oligozoospermic ($P<.01$), and asthenozoospermic men ($P<.01$) compared with pretreatment levels. After treatment, the levels of corrected seminal fructose was also restored in all groups ($P<.05$).

Hormonal Profile

Mean basal hormone levels of infertile subjects and controls are listed in Table 4.

Testosterone and luteinizing hormone Serum T levels were suboptimal in all groups of infertile men. Treatment recovered the levels of T in normozoospermic, oligozoospermic, and asthenozoospermic men significantly ($P<.01$). The serum LH concentration in the control group was 7.94 ± 0.63 mIU/mL. This parameter was significantly lower in all of the infertile men. Treatment significantly increased the level of LH in all of the groups of infertile men.

Follicle-stimulating hormone and prolactin The FSH and PRL levels in the serum of fertile control men were

TABLE 1

Effect of *Withania somnifera* on semen profile of infertile men.

Group	Treatment	Semen volume (mL)	Sperm concentration (10^6/mL)	Sperm count ($\times 10^6$ per ejaculate)	Motility (%)
Control (n = 75)	None	2.84 ± 0.45	79.00 ± 15.37	223.00 ± 52.25	72.67 ± 7.63
Normozoospermic (n = 25)	Pretreatment[a]	2.21 ± 0.43	54.04 ± 8.80	119.37 ± 29.65	54.17 ± 10.18
	Posttreatment[b]	2.65 ± 0.39	71.88 ± 10.70	191.38 ± 42.12	63.54 ± 10.05
Oligozoospermic (n = 25)	Pretreatment[a]	1.86 ± 0.37	9.78 ± 1.89	18.06 ± 4.74	52.5 ± 8.47
	Posttreatment[b]	2.42 ± 0.30	27.32 ± 5.67	65.63 ± 13.52	62.71 ± 9.32
Asthenozoospermic (n = 25)	Pretreatment[a]	2.44 ± 0.45	43.49 ± 8.84	103.37 ± 17.65	16.44 ± 2.91
	Posttreatment	2.54 ± 0.54[NS]	57.6 ± 11.79[b]	141.91 ± 21.95[b]	24.44 ± 4.48[b]

[a] $P < .01$ compared with control (Dunnett test).
[b] $P < .01$ compared with pretreatment (paired *t* test).
[NS] Not significant.

Ahmad. Effect of Withania somnifera on infertile males. Fertil Steril 2009.

5.67 ± 0.91 mIU/mL and 7.10 ± 0.67 ng/mL, respectively. These parameters were significantly elevated in oligozoospermic and asthenozoospermic men. After treatment with *W. somnifera*, FSH and PRL levels were reduced significantly.

The ROC curves suggested that low levels of LH and T were very good indicators of infertility (Figure 1), explaining up to 93.4% and 96.2%, respectively, of cases of infertility (Table 5). Testosterone values <5.81 ng/mL (average for control subjects: 7.09 ng/mL) may indicate infertility with good sensitivity (82.67%) and high specificity (°100%). Similarly, LH values <6.43 mIU/mL (average for control subjects: 7.94 mIU/mL) may indicate infertility with good sensitivity (78.67%) and specificity approximating 100%. However, high values of FSH and PRL were relatively less good indicators of infertility, explaining up to 77.6% and 75.7%, respectively, of infertility cases (Table 5). FSH values >7.19 mIU/mL (average for control subjects: 5.67 mIU/mL) may indicated infertility with low sensitivity (37.33%) and good specificity (99%). Similarly, PRL values >8.0 ng/mL may indicate infertility with low sensitivity (49.33%) but high specificity (99%).

DISCUSSION

The results of this study demonstrate that oxidative stress is associated with reduced antioxidant capacity along with

TABLE 2

Effect of *Withania somnifera* on oxidative biomarkers in seminal plasma of infertile men.

Group	Treatment	Lipid peroxides (nmol MDA/mL)	Protein carbonyl groups (nmol/mg protein)	SOD (U/mg protein)	Catalase (U/mg protein)	Glutathione (mg/dL)
Control (n = 75)		1.74 ± 0.19	2.07 ± 0.37	8.17 ± 0.71	9.18 ± 0.93	1.68 ± 0.15
Normozoospermic (n = 25)	Pretreatment	3.48 ± 0.44[a]	3.14 ± 0.56[a]	6.71 ± 0.88[a]	8.14 ± 1.26*	1.34 ± 0.13[a]
	Posttreatment[b]	2.33 ± 0.29	2.25 ± 0.53	7.36 ± 0.77	10.17 ± 1.77	1.62 ± 0.13
Oligozoospermic (n = 25)	Pretreatment	2.68 ± 0.27[a]	3.74 ± 0.83[a]	5.61 ± 0.90[a]	8.33 ± 1.14*	1.27 ± 0.15[a]
	Posttreatment[b]	2.06 ± 0.27	2.85 ± 0.49	6.93 ± 1.09	9.98 ± 1.01	1.67 ± 0.15
Asthenozoospermic (n = 25)	Pretreatment	3.01 ± 0.55[a]	2.80 ± 0.61[a]	5.29 ± 0.69[a]	6.64 ± 1.00[a]	1.31 ± 0.15[a]
	Posttreatment[b]	2.14 ± 0.22	2.21 ± 0.39	6.49 ± 0.93	8.27 ± 1.99	1.61 ± 0.12

Note: SOD = superoxide dismutase.
* $P < .05$ compared with control (Dunnett test).
[a] $P < .01$ compared with control (Dunnett test).
[b] $P < .01$ compared with pretreatment (paired *t* test).

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TABLE 3

Effect of *Withania somnifera* on seminal plasma levels of antioxidant vitamins and corrected fructose in infertile men.

Group	Treatment	Vitamin A (μg/dL)	Vitamin E (mg/dL)	Vitamin C (mg/dL)	Corrected fructose (mg/mL)
Control (n = 75)		28.61 ± 4.43	0.143 ± 0.012	5.64 ± 0.71	3.63 ± 0.34
Normozoospermic (n = 25)	Pretreatment[a]	17.86 ± 3.02	0.109 ± 0.013	4.18 ± 0.42	2.49 ± 0.30
	Posttreatment	21.73 ± 4.05[b]	0.129 ± 0.019[b]	5.12 ± 0.49[b]	2.77 ± 0.52*
Oligozoospermic (n = 25)	Pretreatment[a]	16.86 ± 3.66	0.089 ± 0.016	4.95 ± 0.77	2.18 ± 0.29
	Posttreatment	19.50 ± 3.41[b]	0.123 ± 0.026[b]	6.03 ± 0.91[b]	2.51 ± 0.29*
Asthenozoospermic (n = 25)	Pretreatment[a]	15.23 ± 2.39	0.078 ± 0.020	5.05 ± 0.88	2.32 ± 0.40
	Posttreatment	17.91 ± 3.06[b]	0.108 ± 0.031[b]	6.13 ± 0.90[b]	2.55 ± 0.41*

[a] $P<.01$ compared with control (Dunnett test).
[b] $P<.01$ compared with pretreatment (paired *t* test).
* $P<.05$ compared with pretreatment (paired *t* test).

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derangement in hormone levels, and that these are negatively correlated with sperm concentration and motility in infertile men (31). Certain individual reports previously showed a direct correlation between seminal oxidative stress and the presence of immature and abnormal spermatozoa due to lipids and protein peroxidations (32). We could trace the reason for oxidative stress to be reduced activity of antioxidant enzymes, namely SOD and catalase, and low glutathione level. We observed that treatment with *W. somnifera* significantly improved the activity of SOD and catalase and the level of glutathione, eventually reducing the levels of LPO and protein carbonyl groups in infertile men. Complementing our findings, earlier studies have reported that *W. somnifera* inhibits lipid peroxidation in stress-induced animals (15). Reduced stress, in effect, might have contributed to the significant improvement in sperm concentration and motility. Although we observed an increase in fructose level with an increase in sperm motility, it remains to be explored if the two could have the cause-and-effect relationship. We have previously reported similar activity in *Mucuna pruriens* (4, 5), but the present results show that *W. somnifera* has better antioxidant properties.

We also observed significantly reduced levels of vitamins A, C, and E in infertile men. Vitamin A is a biologic antioxidant which functions as a detoxifying agent, immunopotentiator, and immunoactivator (33). Similarly, vitamin E (α-tocopherol) contributes to the body's defense system

TABLE 4

Effect of *Withania somnifera* on hormonal profile in serum of infertile men.

Group	Treatment	LH (mIU/mL)	T (ng/mL)	FSH (mIU/mL)	PRL (ng/mL)
Control (n = 75)		7.94 ± 1.00	7.09 ± 0.63	5.67 ± 0.91	7.10 ± 0.67
Normozoospermic (n = 25)	Pretreatment	6.87 ± 0.60[a]	5.80 ± 0.88[a]	6.07 ± 0.69[NS]	7.21 ± 0.72[NS]
	Posttreatment	7.85 ± 0.53[b]	6.65 ± 0.78[b]	5.75 ± 0.60**	6.93 ± 0.67[NS]
Oligozoospermic (n = 25)	Pretreatment	4.02 ± 0.57[a]	3.51 ± 0.56[a]	7.78 ± 0.77[a]	10.57 ± 1.42[a]
	Posttreatment	5.98 ± 0.80[b]	4.94 ± 0.54[b]	6.27 ± 0.76[b]	8.75 ± 1.28[b]
Asthenozoospermic (n = 25)	Pretreatment	3.82 ± 0.59[a]	4.32 ± 0.89[a]	6.49 ± 0.85[a]	7.78 ± 0.82*
	Posttreatment	5.37 ± 0.61[b]	5.23 ± 0.80[b]	5.95 ± 0.96**	7.19 ± 0.82[b]

[a] $P<.01$ compared with control.
[b] $P<.01$ compared with pretreatment.
* $P<.05$ compared with control.
** $P<.05$ compared with pretreatment.
[NS] Not significant.

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TABLE 5

Area under the receiver's operating characteristic curve for different hormones.

Test result variable	Area	SE[a]	Asymptotic Significance[b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
FSH	0.776	0.037	0.000	0.703	0.849
PRL	0.757	0.039	0.000	0.680	0.835
LH	0.934	0.018	0.000	0.899	0.969
T	0.962	0.014	0.000	0.935	0.989

Note: Area under the curve was calculated assuming low values of LH and T and high values of FSH and PRL to indicate positive test for infertility.

[a] Under the nonparametric assumption.

[b] Null hypothesis: true area = 0.5.

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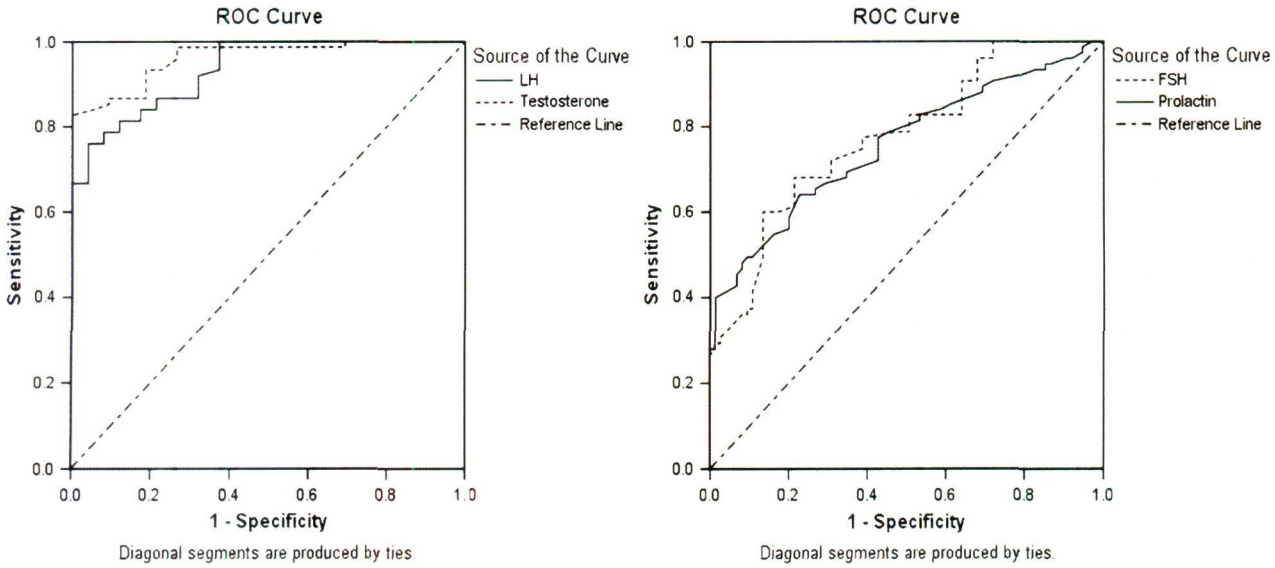
against lipoprotein oxidation and may help in improving sperm motility (34). Healthy fertile men with normal sperm parameters contain adequate amounts of vitamins A and E (35). The impaired antioxidant activity in men with sperm dysfunction may be a reflection of low seminal levels of vitamins A and E (36). Ascorbic acid is a water soluble vitamin and possesses potent ROS-scavenging activity. Seminal plasma is very rich in ascorbic acid content, with a concentration reported to be tenfold higher than in serum (37), highlighting its importance for fertility. Earlier studies also have reported reduced levels of vitamin C in infertile men (38). Treatment with *W. somnifera* improved levels of vitamins A, C, and E, indicating the strength of this herb to protect against infertility due to vitamin loss.

Circulating levels of various sex hormones have been correlated with semen quality parameters (39). Testosterone plays an essential role in spermatogenesis (40). Elevated levels of FSH have been correlated with damage to the seminiferous tubules. FSH has been suggested as a marker of Sertoli cell function and spermatogenesis (41). Our results confirm that LH, T, FSH, and PRL hormone levels could be used as markers of semen quality. We observed decreased levels of LH and T and increased levels of FSH and PRL in

FIGURE 1

Receiver's operating characteristic (ROC) curves showing the diagnostic value of different hormones: the ROC curves were constructed assuming low values of LH and T (**left**) and high values of FSH and PRL (**right**) to indicate positive test for infertility.



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men with poor semen quality. The ROC curve analyses indicated that low values of T and LH were better markers for infertility than high values of FSH and PRL. The sensitivity of both T and LH levels as infertility markers was much higher compared with both FSH and PRL levels. However, the values of all four hormones taken together may help in better decision making regarding the fertility status of an individual. Treatment with *W. somnifera* restored the level of all four hormones. This directly correlates these hormones with semen quality and highlights the capability of *W. somnifera* to restore the level of these hormones. Although direct hormonal supplements have been tried in male infertility treatment, the outcome was very poor (42) and with the cost of certain side effects. Therefore, *W. somnifera* offers a better and safe method of restoring sex hormones in male infertility treatment.

The biologic basis and exact mechanism of action of *W. somnifera* on infertility is not well known, but earlier experimental studies showed that treatment with aqueous extracts of *W. somnifera* induces testicular development and spermatogenesis in immature Wistar rats (17). Moreover, *W. somnifera* has been reported to have several pharmacologic effects, including anthelmintic, narcotic, radiosensitizer, antistressor, adaptogenic, and cardioprotective effects (43). The roots of *W. somnifera* contain several alkaloids, withanolides, and a few flavanoids and reducing sugars (44). More than 20 active constituents have been reported to date in the root of *W. somnifera*, including withaferin A, sitoindosides VII–X, withanosides I–VII, choline, and beta-sitosterol (45). The presence of these compounds may be the reason for diverse effects of *W. somnifera* on semen properties. However, the spermogenic and/or steroidogenic activity of any of these compounds, if any, has not been explored. Therefore, further in-depth studies are needed to explore the individual properties of these active constituents.

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